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(54) Title: METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF ONCOGENES AND TUMOR SUPPRESSOR GENES

(57) Abstract

The invention provides a method for directly effecting transcription of an oncogene or tumor suppressor gene; a method for testing compounds for directly effecting transcription by measuring the product polypeptide produced; and a method for testing compounds for directly effecting transcription by measuring the mRNA produced.

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METHODS OF TRANSCRIPTIONALLY MODULATING EXPRESSION OF
ONCOGENES AND TUMOR SUPPRESSOR GENES

This application is a continuation-in-part of U.S. Serial No. 644,293, filed January 18, 1991, the contents of which are hereby incorporated by reference into the present application.

Background of the Invention

Throughout this application, various publications are referenced by Arabic numerals within parentheses. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The genomes of higher eucaryotic cells contain specific genes that, when activated by any one of several mechanisms, are capable of transforming normal cells to the malignant state. These genes, termed oncogenes, can be loosely defined as genes whose abnormal expression or altered gene product directly contributes to the production of the malignant phenotype. More than 50 such oncogenes have been identified thus far, and current estimates suggest that as many as 200 may be found to exist.

Oncogenes derive from normal cellular progenitors, called proto-oncogenes, many of which code for proteins which

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are involved in c ll division or differentiation. Proto-oncogene encoded products include growth factor-like molecules (int-2), receptors with tyrosine kinase activity (c-erbB2), nuclear proteins (c-myc), membrane
5   bound proteins with GTPase activity (ras), transcription factors (c-jun) and hormone receptors (c-erbA1)(58). The generation of oncogenes from their non-transforming homologues can occur via several different mechanisms; (a) retroviral transduction of proto-oncogenes from a
10   donor cell to host cell during viral replication, placing the donor's proto-oncogene under control of the strong retroviral promoter and resulting in the constitutive over production of a normal protein or in a mutated activation of the protein as a consequence of the
15   transduction event. (b) gene amplification, leading to an increased concentration of the proto-oncogene product (c) binds to DNA or RNA, or chromosomal translocation, resulting in either a novel fusion protein with increased biochemical activity or else overproduction of the
20   translocated cellular gene product by virtue of its placement in proximity to another cellular gene's strong promoter (d) mutation within a proto-oncogene's coding region resulting in a protein product with altered biochemical function(s).
25

Although a relatively large number of oncogenes with potential transforming capabilities exist, the majority have not been implicated in human malignancies. Only about 10 oncogenes have been clearly demonstrated to be
30   involved in human disease.

Recently, a set of cellular genes different from oncogenes, namely tumor suppressor genes or anti-oncogenes, have b en implicated in tumorigenesis. Anti-
35   oncogenes have been defined as genes whose repression,

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inactivati n, dysfunction or loss results in cell
transf rmati n. Tum r suppr ssor genes has been d fined
experimentally as genes whose introducti n, activation or
expression results in the inhibition or suppression of
5   the tumorigenic phenotype. Clearly these definitions are
not mutually exclusive, anti-oncogenes can function as
tumor suppressor genes and vice-versa. The biochemical
function(s) of anti-oncogene products remain to be
elucidated; however, two anti-oncogene products, Rb, the
10   protein product of the cellular retinoblastoma gene, and
p53, have been shown to bind transforming viral proteins.
Loss or inactivation of anti-oncogenes (by allelic
deletion or mutational alteration) may therefore be
involved in tumorigenesis by their failure to interact
15   with and suppress the activity of transforming oncogenes.
In several cases a direct role of tumor suppressor in
control of the cell cycle has been implied.

As stated previously, only a limited number of oncogene
20   and anti-oncogenes have been shown to be present in human
cancers. Within this group, an even smaller number are
thought to play a critical role in a large percentage of
specific cancers or multiple type of neoplasias. The
following is a brief description of those genes which
25   belong to this latter category.

A.   ras: in mammalian species, the ras family of proto-
         oncogenes consists of three closely related members,
         termed H, K, and N-ras. Each codes for a 21 kd
30   protein (p21) with GTP binding and hydrolysis
         activities. The most common mechanism of activation
         of the ras oncogene is a signal point mutation,
         frequently occurring in the protein's twelfth codon,
         which results in a decreased GTPase activity and a
35   concomitant acquisition of transforming potential in

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experimental systems. Furthermore, ras mutations
are found in a large perc nt of cancers, supporting
the supposition that the mutated protein's altered
biochemical function plays a critical role in
5   neoplasias. Specifically, mutated K-ras has been
found in adenocarcinoma of the lung, colon and
pancreas, occurring in 30%, 50% and greater than 70%
of those tumors, respectively (1,2)

10   K-ras mutations have also been reported in
                over 20% of melanoma tumor samples
                recently examined (3). Between 50 and 70%
                of the different tumor types are
                refractory to current methods of therapy
15   and eventually prove fatal.

        In addition to the preponderance of mutated ras
        protein in human cancer, a number of reports
        suggests a link between overexpression of the normal
20   ras gene and neoplastic transformation (4,5,6).
        Regardless of the mechanism of activation, the ras
        oncogene is the most implicated of all oncogenes in
        its wide range and high percentage that is found in
        a variety of human neoplasias.
25
    B. erbB2 (neu): The erbB2 gene encodes a 185 Kd
        transmembrane protein-tyrosine kinase which closely
        resembles the EGF receptor. Amplification of the
        erbB2 gene has been found to occur in 30% of breast
30   cancer (7). Amplification is found in both early
        and late stages of disease, and is maintained in the
        metastases. Furthermore, there is a direct
        correlation between gene amplification and
        overexpression of the erbB2 protein, although
35   numerous examples of tumors which overexpress

5

prot in in the absence of gene amplification also
exist. Overexpr ssion of the erbB2 protein also
ccurs in 30% of ovarian cancers. In both tumor
types, erbB2 overexpression correlates with poor
patient prognosis, with a shorter time to relapse as
well as a decreased overall survival rate.

In experimental systems, overexpression of the
normal human erbB2 gene in murine fibroblasts to
levels similar to those in human breast and ovarian
cancers have been shown to induce cell
transformation (8,9). In addition, erbB2
overexpression in the human MCF-7 breast cancer line
allows these cells to grow as tumors in nude mice.
Furthermore, monoclonal antibodies to human erbB2
have specific anti-proliferative effects in vitro of
human breast tumor cells which overexpress erbB2
(10). These and other data strongly suggest that
the erbB2 gene plays an important role in the onset
and progression of breast and other cancers.

C.   p53: The first host protein shown to bind
specifically to the product of a DNA tumor virus
oncogene (SV40 large T-antigen) was p53 (11,12).

Recent studies examining the genetic alterations
which occur during the development of colorectal
tumors have indicated a common mutation to be
deletions of the short arm of chromosome 17, the
region where the p53 gene is located. Chromosome
17p deletions are often late events associated with
the transition from the benign (adenomatous) to the
malignant (carcinomatous) state (13). In a vast
majority of colorectal carcinomas examined, allelic
deletions of 17p were found to occur. The remaining

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p53 allele was shown to contain point mutations
suggesting that p53 gene mutations may be involved
in colorectal neoplasias, perhaps through
inactivation of a tumor suppressor function of the
5   wild type p53 gene (14). Allelic loss of p53
(chromosome 17p) has also been observed in a large
number of breast and lung tumors analyzed. In a
study involving a limited number of hepatocellular
carcinoma-derived cell lines, six of seven showed
10   p53 abnormalities (15) suggesting a general
mechanism of p53 inactivation in several different
human neoplasias (16,17,18) Recently specific
mutations in p53 have been found in a large
percentage of cases of malignant melanoma.
15

The specific cellular function of p53 has not yet
been determined, although several lines of evidence
suggest a role in the regulation of DNA replication
(19). Loss of one p53 allele followed by mutational
20   inactivation of the remaining allele could therefore
result in a mutated p53 protein incapable of
carrying out its normal function, one proposed
function being tumor suppressor activity. There is
increasing evidence that mutated p53 may play an
25   active role in transformation suggesting that p53
may act as either an oncogene or an anti-oncogene
depending on whether it is mutant or wild-type.

Pharmaceuticals which increase or decrease the expression
30   of oncogenes, anti-oncogenes or tumor suppressor genes
will have important clinical applications for the
treatment of various neoplasias. We describe herein a
method for discovery of compounds which modulate the
expression of these genes and describe the use of such
35   compounds. The general approach is to screen compound

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libraries for substances which incr as or d crease
xpressi n f ncogenes, anti- ncogenes, tum r suppressor
genes r proto-oncogenes.

5   The expression of a specific gene can be regulated at any
step in the process of producing an active protein.
Modulation of total protein activity may occur via
transcriptional, transcript-processing, translational or
post-translational mechanisms.   Transcription may be
10   modulated by altering the rate of transcriptional
initiation or the progression of RNA polymerase (28).
Transcript-processing may be influenced by circumstances
such as the pattern of RNA splicing, the rate of mRNA
transport to the cytoplasm or mRNA stability. This
15   invention concerns the use of molecules which act by
modulating the in vivo concentration of their target
proteins via regulating gene transcription.   The
functional properties of these chemicals are distinct
from previously described molecules which also affect
20   gene transcription.

Researchers have documented the regulation of
transcription in bacteria by low molecular weight
chemicals (20, 21).   Extracellular xenobiotics, amino
25   acids and sugars have been reported to interact directly
with an intracellular proteinaceous transcriptional
activator or repressor to affect the transcription of
specific genes.

30   Transcriptional regulation is sufficiently different
between procaryotic and eucaryotic organisms so that a
direct comparison cannot readily be made. For example,
procaryotic cells lack a distinct membrane bound nuclear
compartment. Furthermore the structure and organization
35   of procaryotic DNA elements responsible for initiation of

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transcripti n diff r mark dly from thos of eucaryotic
cells.

The eucaryotic transcriptional unit is much more complex
than its procaryotic counterpart and consists of
additional elements which are not commonly found in
bacteria, including enhancers and other cis-acting DNA
sequences (22, 23). Procaryotic transcription factors
most commonly exhibit a "helix-turn-helix" motif in the
DNA binding domain of the protein (24, 25). Eucaryotic
transcriptional factors frequently contain a "zinc
finger" (25, 26), "helix-loop-helix" or a "leucine
zipper" (27) in addition to sometimes possessing the
"helix-turn-helix" motif (28).   Furthermore, several
critical mechanisms at the post-transcriptional level
such as RNA splicing and polyadenylation are not
typically found in procaryotic systems (29, 30).

In higher eucaryotes, modulation of gene transcription in
response to extracellular factors can be regulated in
both a temporal and tissue specific manner (31). For
example, extracellular factors can exert their effects
by directly or indirectly activating or inhibiting
tissue specific transcription factors (31, 32).

Modulators of transcription factors involved in direct
regulation of gene expression have been described, and
include those extracellular chemicals entering the cell
passively and binding with high affinity to their
receptor-transcription factors. This class of direct
transcriptional modulators include steroid hormones and
their analogs, thyroid hormones, retinoic acid, vitamin
D_3 and its derivatives, and dioxins, a chemical family of
polycyclic aromatic hydrocarbons (26, 33, 34).

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Di xins ar   molecules g nerally known to modulate
transcripti n,   h w ver,   dioxins   bind   to
naturally-occurring r ceptors which respond normally to
xenobiotic agents via transcriptionally activating the
5   expression of cytochrome P450, part of an enzyme involved
in detoxification. Similarly, plants also have naturally
occurring receptors to xenobiotics to induce defense
pathways. For example, the fungal pathogen Phytophthora
megasperma induces an anti-fungal compound in soybeans.
10   Such molecules which bind to the defined ligand binding
domains of such naturally occurring receptors are not
included on the scope of this invention.

The clinical use of steroid hormones, thyroid hormones,
15   vitamin D_3 and their analogs demonstrates that agents
which modulate gene transcription can be used for
beneficial effects, although these agents can exhibit
significant adverse side effects. Obviously, analogs of
these agents could have similar clinical utility as their
20   naturally occurring counterparts by binding to the same
ligand binding domain of such receptors. These types of
molecules do not fall within the scope of this invention
because they function by binding to the ligand-binding
domain of a receptor normally associated with a defined
25   physiological effect.

Indirect transcriptional regulation involves one or more
signal transduction mechanisms. This type of regulation
typically involves interaction with a trans-membrane
30   signal transducing protein, the protein being part of a
multistep intracellular signaling pathway, the pathway
ultimately modulating the activity of nuclear
transcription factors.   This class of indirect
transcriptional modulators include polypeptide growth
35   factors such as platelet-derived growth factor, epidermal

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growth fact r, cyclic nucl otid analogs, and mitogenic
tum r prom ters such as PMA (35, 36, 37).

It is well documented that a large number of chemicals,
5　both　organic　and　inorganic,　e.g.　metal　ions,　can
non-specifically　modulate　transcription.　Most　heavy
metals modulate gene expression through receptors in a
mechanism similar to that employed by dioxin, steroid
hormones, vitamin D3 and retinoic acid.

10

Researchers have used nucleotide analogs in methods to
non-specifically modulate transcription.　The mechanism
involves incorporating nucleotide analogs into nascent
mRNA　or　non-specifically　blocking　mRNA　synthesis.
15　Similarly, researchers have used alkylating agents, e.g.
cyclophosphamide,　or　intercalating　agents,　e.g.
doxorubicin, to non-specifically inhibit transcription.

Moreover, chemical inhibitors of hydroxymethyl-glutaryl
20　CoA reductase, e.g. lovastatin, are known to indirectly
modulate　transcription　by　increasing　expression　of
hepatic　low　density　lipoprotein　receptors　as　a
consequence of lowered cholesterol levels.

25　Signal　effector　type　molecules　such　as　cyclic　AMP,
diacylglycerol,　and　their　analogs　are　known　to
non-specifically regulate transcription by acting as part
of a multistep protein kinase cascade reaction.　These
signal　effector　type　molecules　bind　to　domains　on
30　proteins which are thus subject to normal physiological
regulation by low molecular weight ligands (38, 39).

The specific use of sterol regulatory elements from the
LDL receptor gene to control expression of a reporter
35　gene has recently been documented in PCT/US88/10095. One

aspect of PCT/US88/10095 deals with the use of specific
ster l regulatory lements c upled to a reporter as a
means to scr en f r drugs capable of stimulating cells to
synthesize the LDL receptor. PCT/US88/10095 describes
5   neither the concept of simultaneously screening large
numbers of chemicals against multiple target genes nor
the existence of transcriptional modulators which (a) do
not naturally occur in the cell, (b) specifically
transcriptionally modulate expression of the gene
10 encoding the oncogene or tumor suppressor gene product,
and (c) binds to DNA or RNA, or bind to DNA or RNA or
bind to a protein through a domain of such protein which
is not a defined ligand binding domain of a nuclear,
transcriptionally activating receptor which naturally
15 occurs in the cell, the binding of a ligand to which
ligand binding domain is normally associated with a
defined physiological effect. The main focus of
PCT/US88/10095 is the use of the sterol regulatory
elements from the LDL receptor as a means to inhibit
20 expression of toxic recombinant biologicals.

The use of molecules to specifically modulate
transcription of oncogenes and tumor suppressor genes as
described herein has not previously been reported and its
25 use will bring surprise since available literature does
not propose the use of a molecule, as described, in a
method to specifically modulate transcription. Instead,
the available literature has reported methods which
define domains of transcriptional regulating elements of
30 a gene.

Further, the practice of using a reporter gene to analyze
nucleotide sequences which regulate transcription of a
gene-of-interest is well documented. The demonstrated
35 utility of a reporter gene is in its ability to define

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domains f transcripti nal regulat ry elements of a g n -
f-interest. Rep rter genes which express proteins, .g.
luciferase, are widely utilized in such studies.
Luciferases expressed by the North American firefly,
5   <u>Photinus pyralis</u> and the bacterium, <u>Vibrio fischeri</u> were
first described as transcriptional reporters in 1985 (40,
41). Reporter genes have not been previously used to
identify compounds which (a) do not naturally occur in
the cell, (b) specifically transcriptionally modulate
10   expression of the gene encoding the oncogene or the tumor
suppressor gene product, and (c) binds to DNA or RNA, or
bind to a protein at a site on such protein which is not
a ligand-binding domain of a receptor which naturally
occurs in the cell, the binding of a ligand to which
15   ligand-binding domain is normally associated with a
defined physiological or pathological effect.

A method to define domains of transcriptional regulating
elements of a gene-of-interest typically has also
20   involved use of phorbol esters, cyclic nucleotide
analogs, concanavalin A, or steroids, molecules which are
commonly known as transcriptional modulators. However,
available literature shows that researchers have not
considered using a transcription screen to identify
25   specific transcriptional modulators. Apparently, success
would be unlikely in doing so, however, we have
demonstrated herein that this is not the case.

There is utility in developing the method of
30   transcriptional modulation of oncogenes and tumor
suppressor genes by using such molecule as described
herein. This method will allow the development of novel
pharmaceuticals and circumvent many of the problems
associated with the therapeutic use of recombinant
35   biological factors where clinical the use of protein

fact rs is r levant.

Problems associat d with the therapeutic use f
recombinant biological factors include the technical
5   difficulties of large scale protein purification, the
high costs of protein production, the limited shelf-life
of most proteins and in some cases a short biological
half-life of the administered protein in the organism.
Additionally, therapeutic delivery of proteins normally
10   requires injection. The method described herein provides
a means of upregulating the expression of proteins which
are not readily amenable to administration as injectable
biologicals.

15   Furthermore, molecules specifically regulating the
activity of one member of a group of closely related
proteins are difficult to identify. Bioactive molecules,
structurally related at the protein level, may possess
distinct regulatory elements at the DNA level which
20   control their expression. Thus, molecules such as the
chemical transcriptional modulators defined herein can
provide a greater opportunity for specifically modulating
the activity of structurally related proteins.

25   Finally, the molecules described herein may also serve to
mimic normal physiological response mechanisms, typically
involving the coordinated expression of one or more
groups of functionally related genes. Therefore,
determining whether a molecule can specifically
30   transcriptionally modulate the expression of an oncogene
or tumor suppressor gene and the ultimate clinical use of
the molecule provides a therapeutic advantage over the
use of single recombinant biologicals, or drugs which
bind directly to the final target protein encoded by the
35   gene-of-interest.

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Summary of the Invention

The invention provides a method of directly
transcriptionally modulating the expression of an
5   oncogene or a tumor suppressor gene, the expression of
which is associated with a defined physiological or
pathological effect within a multicellular organism.
This method comprises contacting a cell, which is capable
of expressing the gene, with a molecule at a
10   concentration effective to transcriptionally modulate
expression of the gene and thereby affect the level of
the oncogene or the tumor suppressor gene product(s)
encoded by the gene which is expressed by the cell. In
this method the molecule (a) does not naturally occur in
15   the cell and (b) specifically transcriptionally modulates
expression of the oncogene or the tumor suppressor gene,
and (c) binds to DNA or RNA, or binds to a protein at a
site on such protein which is not a ligand-binding domain
of a receptor which naturally occurs in the cell, the
20   binding of a ligand to which ligand-binding domain is
normally associated with a defined physiological or
pathological effect.

The invention further provides for a method of
25   determining whether a molecule not previously known to be
a modulator of protein biosynthesis is capable of
transcriptionally modulating the expression of an
oncogene or a tumor suppressor gene. This method
comprises contacting a sample which contains a predefined
30   number of cells with a predetermined amount of a molecule
to be tested, each such cell comprising DNA consisting
essentially of (i) a modulatable transcriptional
regulatory sequence of the oncogene or the tumor
suppressor gene, (ii) a promoter of the oncogene or the
35   tumor suppressor gene, and (iii) a DNA sequence encoding

a polyp ptide other than th   ncog n   or the tumor
suppress r, which polyp ptide is capable of producing a
detectable signal, and which DNA sequence is coupled to,
and under the control of, the promoter, under conditions
5   such that the molecule, if capable of acting as a
transcriptional modulator of the oncogene or the tumor
suppressor gene, causes a measurable detectable signal to
be produced by the polypeptide so expressed. The amount
of the signal produced, is quantitatively determined and
10   the amount so determined compared with the amount of
produced signal detected in the absence of any molecule
being tested or upon contacting the sample with any other
molecule. So as to identify the molecule as one which
causes a change in the detectable signal produced by the
15   polypeptide so expressed, and thus identify the molecule
as a molecule capable of transcriptionally modulating the
expression of the oncogene or the tumor suppressor gene.

Additionally the invention provides a method of
20   determining whether a molecule not previously known to be
a modulator of protein biosynthesis is capable of
transcriptionally modulating the expression of an
oncogene or a tumor suppressor gene. The method
comprises contacting a sample which contains a predefined
25   number of cells with a predetermined amount of a molecule
to be tested. The cells so contacted comprise   DNA
consisting essentially of (i) a modulatable
transcriptional regulatory sequence of the gene encoding
the oncogene or the tumor suppressor gene, (ii) a
30   promoter of the oncogene or the tumor suppressor gene,
and (iii) a reporter gene, which expresses a polypeptide,
coupled to, and under the control of, the promoter, under
conditions such that the molecule, if capable of acting
as a transcriptional modulator of the oncogene or the
35   tumor suppressor gene, causes a measurable change in the

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am unt of th polyp ptide produced. The am unt of th
polypeptide s produced, is quantitatively determined and
the amount so determined is compared with the amount of
polypeptide produced in the absence of any molecule being

5   tested or upon contacting the sample with any other
molecule. So as to identify the molecule as one which
causes a change in the amount of the polypeptide
expressed, and thus identify the molecule as a molecule
capable of transcriptionally modulating the expression of

10   the oncogene or the tumor suppressor gene.

A method of determining whether a molecule not previously
known to be a modulator of protein biosynthesis is
capable of transcriptionally modulating the expression of

15   an oncogene or a tumor suppressor gene, is also provided.
This method comprises contacting a sample which contains
a predefined number of cells with a predetermined amount
of a molecule to be tested. Each of the cells so
contacted comprises DNA consisting essentially of (i) a

20   modulatable transcriptional regulatory sequence of the
oncogene or the tumor suppressor gene, (ii) a promoter of
the oncogene or the tumor suppressor gene, and (iii) a
DNA sequence transcribable into mRNA coupled to and under
the control of, the promoter. The contacting is effected

25   under conditions such that the molecule, if capable of
acting as a transcriptional modulator of the oncogene or
the tumor suppressor gene, causes a measurable difference
in the amount of mRNA transcribed from the DNA sequence.
The amount of the mRNA produced is quantitatively

30   determined and the amount so determined compared with the
amount of mRNA detected in the absence of any molecule
being tested or upon contacting the sample with any other
molecule so as to identify the molecule as one which
causes a change in the detectable mRNA amount of, and

35   thus identify the molecule as a molecule capable of

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transcriptionally modulating th expression of the
oncog ne or tumor suppressor g ne.

A screening method which comprises separately contacting
5   each of a plurality of substantially identical samples,
each sample containing a predefined number of cells under
conditions such that contacting is affected with a
predetermined amount of each different molecule to be
tested, is also provided.
10

Further provided is a method of essentially
simultaneously screening molecules to determine whether
the molecules are capable of transcriptionally modulating
one or more oncogenes or tumor suppressor genes according
15   to the methods mentioned above.

Further provided is a method for directly
transcriptionally modulating in a multicellular organism
the expression of an oncogene or a tumor suppressor gene,
20   the expression of which is associated with a defined
physiological or pathological effect in the organism.
This method comprises administering to the organism a
molecule at a concentration effective to
transcriptionally modulate expression of the gene and
25   thus affect the defined physiological or pathological
effect. In this method the molecule (a) does not
naturally occur in the organism, (b) specifically
transcriptionally modulates expression of the oncogene or
the tumor suppressor gene, and (c) binds to DNA or RNA,
30   or binds to a protein at a site on such protein which is
not a ligand-binding domain of a receptor which naturally
occurs in the cell, the binding of a ligand to which
ligand-binding domain is normally associated with a
defined physiological or pathological effect.

Brief Description of the Figures

Figure 1 is a view f the mammalian expression shuttle vector pUV102 with its features. The mammalian expression shuttle vector was designed to allow the construction of the promoter-reporter gene fusions and the insertion of a neomycin resistance gene coupled to the herpes simplex virus thymidine kinase promoter (TK-NEO).

Figure 2 is a partial restriction enzyme cleavage map of the plasmid pDO432 which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 3 is a partial restriction enzyme cleavage map of the plasmid pSVLuci which contains the luciferase gene from the firefly, Photinus pyralis.

Figure 4 is a partial restriction enzyme cleavage map of the plasmid pMLuci which contains the luciferase gene of the firefly, Photinus pyralis and the mouse mammary tumor virus long terminal repeat.

Figure 5 provides the nucleotide sequences of six oligonucleotides, pUV-1 through pUV-6, which were annealed, ligated, and inserted into the SalI/EcoR1 sites of the plasmid pTZ18R.

Figure 6 is a diagrammatic representation of the construction of the plasmid pUV001 from the plasmids pTZ18R and pBluescript KS(+).

Figure 7 is a diagrammatic representation of the construction of the plasmid pUV100 from the plasmid pUV001 and two DNA fragments, the XbaI/XmaI fragment from

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pMLuci and the XmaI/BamHI fragment from pMSG.

Figure 8 is a diagrammatic repres ntation of the construction of the plasmid pUV100-3 from the plasmid pUV100 and a 476 b fragment containing a dimeric SV40 polyadenylation site.

Figure 9 is a diagrammatic representation of the construction of the plasmids pUV102 and pUV103 from the plasmid pUV100-3 and D-link oligonucleotides and the plasmid pUV100-3 and R-link oligonucleotides, respectively.

Figure 10 provides the nucleotide sequences of oligos 1-4 used for the construction of a synthetic HSV-thymidine kinase promoter and provides a diagrammatic representation of the HSV-TK promoter.

Figure 11 is a diagrammatic representation of the construction of the plasmid pTKL100 which contains the luciferase gene from the firefly, Photinus pyralis and the HSV-TK promoter sequence.

Figure 12 is a diagrammatic representation of the construction of the plasmid pTKNEO which contains the neo gene, from about 3.5 kb NheI/XmaI fragment from pTKL100, and the about 0.9 kb BstBI/BglII fragment containing the neo coding region from pRSVNEO.

Figure 13 is a diagrammatic representation of the construction of the plasmid pTKNEO2 from the plasmid pTKNEO and the oligonucleotides Neo 1 and 2.

Figure 14 is a diagrammatic representation of the construction of the plasmid pTKNEO3 from the plasmid

20

PTKNEO2 and about 0.9 kb EcoR1/SalI fragm nt from
pMC1NEO.

Figure 15 is a partial restriction enzyme cleavage map of
5   the plasmid pNEU106 which contains neu upstream sequences
fused to the luciferase coding region.

Figure 16 is a partial restriction enzyme cleavage map of
the plasmid pKRAS106 which contains K-ras upstream
10   sequences fused to the luciferase gene from the firefly,
Photinus pyralis.

Figure 17 is a partial restriction enzyme cleavage map of
the plasmid pHRAS106 which contains human H-ras upstream
15   sequences fused to the luciferase gene from the firefly,
Photinus pyralis.

Figure 18 is a partial restriction enzyme cleavage map of
the plasmid pNRAS106 which contains human N-ras upstream
20   sequences fused to the luciferase gene from the firefly,
Photinus pyralis.

Figure 19 is a diagrammatic representation of the phl
gene. Shown are EcoRI sites and genomic fragments used
25   in defining the phl promoter.

Figure 20 is a diagrammatic representation of the various
phl-luciferase fusion plasmids used to define the phl
promoter. Plasmid 4 was used to create the stably
30   transfected phl reporter cell line.

Figure 21 is a partial restriction enzyme cleavage map of
the plasmid pP531106 which contains human p53 P1 promoter
upstream sequences fused to the luciferase gene from the
35   firefly, Photinus pyralis.

21

Figure 22 is a partial restriction enzym cleavag map f
the plasmid pP532106 which contains human p53 P2 pr moter
upstream s quenc s fus d t the luciferas g n from the
firefly, _Photinus pyralis_.

Figure 23 is a partial restriction enzyme cleavage map of
the plasmid pCM106 which contains the Cytomegalovirus
immediate early promoter fused to the luciferase gene
from the firefly, _Photinus pyralis_.

Figure 24 is a partial restriction enzyme cleavage map of
a human _neu_ minigene which contains the _neu_ upstream
regulatory elements fused to the _neu_ cDNA.

Figure 25 is a partial restriction enzyme cleavage map of
a human K-_ras_ minigene which contains the K-_ras_ upstream
regulatory elements fused to the neu cDNA.

Figure 26 is a graphical representation of the decay of
reporter gene signal after treatment of cells with
Actinomycin D. Plotted is relative intensity of the
signal versus time after ActD addition.

Figure 27 is an autoradiogram of a Southern blot showing
the correct luciferase vector integration of five
independently isolated H-_ras_ transfectants. Lanes 1 and
2 are plasmid controls. The expected result is a single
band of the same molecular weight as the control.

Figure 28 is an autoradiogram of PCR reactions detecting
varying amounts of M-CSF mRNA and a constant amount of
lambda DNA.

Figure 29 is an interpretation of the data presented in
Figure 28. Relative band intensity of the M-CSF band is

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pl tted against th t tal RNA.

Figure 30 is an ethidium bromide stained gel showing the
reaction products of PCR reactions designed to
specifically detect N-ras, neu, K-ras and H-ras mRNAs.
10 and 0.4 ng of U5637 total RNA was used. In each
sample a sample of phage lambda DNA and lambda control
oligos was added to control for the efficiency of the PCR
reaction. This is the uppermost, consistent band in each
lane. In each case the oligos specifically amplifies a
DNA fragment of the correct molecular weight. A few
extra bands of unknown origin appear in the c-erbB2 lanes
and in the lane representing the highest concentration of
H-ras mRNA.

Figure 31 is a quality assurance analysis of a high
throughput screen measuring the ratios of negative values
at various positions within a plate. The expected value
is 1.0.

Figure 32 is a quality assurance analysis of a high
throughput screen measuring a coefficient of variance for
the negative controls on a number of plates. Values less
than 10 are acceptable.

Figure 33 is a quality assurance analysis of a high
throughput screen measuring a coefficient of variance for
the positive controls on a number of plates. Values less
than 10 are acceptable.

Figure 34 is a quality assurance analysis of a high
throughput screen measuring a response of a reporter cell
line to three different concentrations of a compound
known to induce transcription.

23

Detailed Description of the Invention

As used in this applicati n, the f llowing words r phrases have the meanings specified.

5

Antisense nucleic acid means an RNA or DNA molecule or a chemically modified RNA or DNA molecule which is complementary to a sequence present within an RNA transcript of a gene.

10

Directly transcriptionally modulate the expression of a gene means to transcriptionally modulate the expression of the gene through the binding of a molecule to (1) the gene (2) an RNA transcript of the gene, or (3) a protein

15 which binds to (i) such gene or RNA transcript, or (ii) a protein which binds to such gene or RNA transcript.

A gene means a nucleic acid molecule, the sequence of which includes all the information required for the

20 normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

Indirectly transcriptionally modulate the expression of

25 a gene means to transcriptionally modulate the expression of such gene through the action of a molecule which cause enzymatic modification of a protein which binds to (1) the gene or (2) an RNA transcript of the gene, or (3) protein which binds to (i) the gene or (ii) an RNA

30 transcript of the gene. For example, altering the activity of a kinase which subsequently phosphorylates and alters the activity of a transcription factor constitutes indirect transcript modulation.

35 Ligand means a molecule with a molecular weight of less

24

than 5,000, which binds t a transcripti n fact r for a
gene. The binding of the ligand t the transcription
factor transcriptionally modulates the expression of the
gene.

5

Ligand binding domain of a transcription factor means the
site on the transcription factor at which the ligand
binds.

10   Modulatable transcriptional regulatory sequence of a gene
means a nucleic acid sequence within the gene to which a
transcription factor binds so as to transcriptionally
modulate the expression of the gene.

15   Oncogene means a normal cellular gene which has been
activated by one of several possible mechanisms,
including point mutations, translation, amplification or
overexpression contributes to the malignant phenotype of
a cancer cell.

20

Receptor means a transcription factor containing a ligand
binding domain.

Specifically transcriptionally modulate the expression of
25   a gene means to transcriptionally modulate the expression
of such gene alone, or together with a limited number of
other genes.

Transcription means a cellular process involving the
30   interaction of an RNA polymerase with a gene which
directs the expression as RNA of the structural
information present in the coding sequences of the gene.
The process includes, but is not limited to the following
steps: (1) the transcription initiation, (2) transcript
35   elongation, (3) transcript splicing, (4) transcript

capping, (5) transcript termination, (6) transcript
polyadenylation, (7) nuclear export of the transcript,
(8) transcript diting, and (9) stabilizing the
transcript.

Transcription factor for a gene means a cytoplasmic or
nuclear protein which binds to (1) such gene, (2) an RNA
transcript of such gene, or (3) a protein which binds to
(i) such gene or such RNA transcript or (ii) a protein
which binds to such gene or such RNA transcript, so as to
thereby transcriptionally modulate expression of the
gene.

Transcriptionally modulate the expression of a gene means
to change the rate of transcription of such gene.

Triple helix means a helical structure resulting from the
binding of one or more oligonucleotide to double stranded
DNA.

Tumor suppressor gene means a normal cellular gene, the
loss of whose function contributes to the malignant
phenotype of a cancer cell.

The invention provides a method of directly
transcriptionally modulating the expression of an
oncogene or a tumor suppressor gene, the expression of
which is associated with a defined physiological or
pathological effect within a multicellular organism.
This method comprises contacting a cell, which is capable
of expressing the gene, with a molecule at a
concentration effective to transcriptionally modulate
expression of the gene and thereby affect the level of
the oncogene or the tumor suppressor gene product encoded
by the gene which is expressed by the cell. In this

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method the m l cule (a) do s not naturally ccur in the
cell, (b) specifically transcriptionally modulates
expression of the oncogene or the tumor suppressor gene,
and (c) binds to DNA or RNA, or binds to a protein at a

5   site on such protein which is not a ligand-binding domain
of a receptor which naturally occurs in the cell, the
binding of a ligand to which ligand-binding domain is
normally associated with a defined physiological or
pathological effect.

10

In one embodiment the molecule does not naturally occur
in any cell of a lower eucaryotic organism such as yeast.

In a preferred embodiment, the molecule does not

15   naturally occur in any cell, whether of a multicellular
or a unicellular organism. Alternatively, the molecule
is naturally occurring, but not normally found in the
cell. In a presently more preferred embodiment, the
molecule is not a naturally occurring molecule, e.g. is

20   a chemically synthesized entity.

The cell in the above methods may be a cell of the
multicellular organism, such as an animal cell, e.g. a
human cell.

25

The method of the invention permits modulation of the
transcription of the gene which results in upregulation
or downregulation of expression of the oncogene or the
tumor suppressor gene, depending on the identity of the

30   molecule which contacts the cell.

In one embodiment the molecule binds to a modulatable
transcription sequence of the gene. For example, the
molecule may bind to a promoter region upstream of the

35   coding sequence encoding the oncogene or the tumor

27

suppress r gen .

In ne embodiment f the meth d f the invention the
molecule comprises an antisense nucleic acid which is
5   complementary to a sequence present in a modulatable,
transcriptional sequence. The molecule may also be a
double-stranded nucleic acid or a nucleic acid capable of
forming a triple helix with a double-stranded DNA.

10   In accordance with the method of this invention, the
oncogene may be a mutant c-neu gene, a mutant c-K-ras
gene, a mutant c-H-ras gene, a mutant c-N-ras gene, a
phl-abl gene fusion, a mutant myc gene, a mutant fms
gene, a mutant erbA gene, a retroviral oncogene or a
15   mutant p53 gene. The tumor suppressor gene may be a p53
gene, a retinoblastoma gene, a Wilms' tumor gene, a
neurofibromatosis type 1 gene, a DCC gene, an erbA gene,
or an adenomatous polyposis coli gene.

20   The invention further provides a method of determining
whether a molecule not previously known to be a modulator
of protein biosynthesis is capable of transcriptionally
modulating the expression of an oncogene or the tumor
suppressor gene. This method comprises contacting a
25   sample which contains a predefined number of cells with
a predetermined amount of a molecule to be tested, each
such cell comprising DNA consisting essentially of (i) a
modulatable transcriptional regulatory sequence of the
oncogene or the tumor suppressor gene, (ii) a promoter of
30   the oncogene or the tumor suppressor gene, and (iii) a
DNA sequence encoding a polypeptide other than the
oncogene or the tumor suppressor gene product, which
polypeptide is capable of producing a detectable signal
and which DNA sequence is coupled to, and under the
35   control of, the promoter, under conditions such that the

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mol cul , if capable f acting as a transcriptional
modulator of the oncogene or the tumor suppressor gene,
causes a measurable detectable signal to be produced by
the polypeptide so expressed. The amount of the signal
5 produced is quantitatively determined and the amount so
determined compared with the amount of produced signal
detected in the absence of any molecule being tested or
upon contacting the sample with any other molecule so as
to identify the molecule as one which causes a change in
10 the detectable signal produced by the polypeptide so
expressed, and identify the molecule as a molecule
capable of transcriptionally modulating the expression of
the oncogene or the tumor suppressor gene.

15 Additionally, the invention provides a method of
determining whether a molecule not previously known to be
a modulator of protein biosynthesis is capable of
transcriptionally modulating the expression of an
oncogene or a tumor suppressor gene. The method
20 comprises contacting a sample which contains a predefined
number of cells with a predetermined amount of a molecule
to be tested. The cells comprise DNA consisting
essentially of (i) a modulatable transcriptional
regulatory sequence of the gene encoding the oncogene or
25 the tumor suppressor gene, (ii) a promoter of the
oncogene or the tumor suppressor gene, and (iii) a
reporter gene, which expresses a polypeptide, coupled to,
and under the control of, the promoter, under conditions
such that the molecule, if capable of acting as a
30 transcriptional modulator of the oncogene or tumor
suppressor gene, causes a measurable change in the amount
of the polypeptide produced. The amount of the
polypeptide is quantitatively determined and compared
with the amount of polypeptide produced in the absence of
35 any molecul b ing tested or upon contacting the sample

29

with any ther m lecule so as to identify the molecule as
 ne which causes a change in th amount of the
p lypeptid expressed, and thus identify th m lecule as
a molecule capable of transcriptionally modulating the
expression of the oncogene or the tumor suppressor gene.

A method of determining whether a molecule not previously
known to be a modulator of protein biosynthesis is
capable of transcriptionally modulating the expression of
an oncogene or a tumor suppressor gene, is also provided.
This method comprises contacting a sample which contains
a predefined number of cells with a predetermined amount
of a molecule to be tested. Each of the cells comprise
DNA consisting essentially of (i) a modulatable
transcriptional regulatory sequence of the oncogene or
the tumor suppressor gene, (ii) a promoter of the
oncogene or tumor suppressor gene, and (iii) a DNA
sequence transcribable into mRNA coupled to and under the
control of, the promoter, under conditions such that the
molecule, if capable of acting as a transcriptional
modulator of the oncogene or the tumor suppressor gene,
causes a measurable difference in the amount of mRNA
transcribed from the DNA sequence. The amount of the
mRNA produced is quantitatively determined the amount so
determined compared with the amount of mRNA detected in
the absence of any molecule being tested or upon
contacting the sample with any other molecule so as to
identify the molecule as one which causes a change in the
detectable mRNA amount of, and thus identify the molecule
as a molecule capable of transcriptionally modulating the
expression of the oncogene or the tumor suppressor gene.

In the above methods the sample preferably comprises
cells in monolayers or suspension. The cells are
typically animal cells, e.g. human cells. The predefined

number of cells is preferably from about 1 t ab ut 5 X
10^5 cells, or from about 2 X 10^2 t ab ut 5 X 10^4 cells.
Th predetermined am unt or concentration of the molecule
to be tested is typically based upon the volume of the
5   sample, or be from about 1.0 pM to about 20 μM, or from
about 10μM to about 500μM. The contacting may be
effected from about 1 to about 24 hours, or from about 2
to about 12 hours, it may also be effected with more than
one predetermined amount of the molecule to be tested.
10

The molecule to be tested may be a purified molecule.
Additionally the modulatable transcriptional regulatory
sequence may comprises a cloned genomic regulatory
sequence. The DNA referred to in the above methods may
15   consist essentially of more than one modulatable
transcriptional regulatory sequence. The DNA sequence
encoding the polypeptide may be inserted downstream of
the promoter of the oncogene or tumor suppressor gene by
homologous recombination.
20

In these methods the polypeptide may be a luciferase,
chloramphenicol acetyltransferase, β glucuronidase, β
galactosidase, neomycin phosphotransferase, alkaline
p h o s p h a t a s e   o r   g u a n i n e   x a n t h i n e
25   phosphoribosyltransferase. The polypeptide may be
capable of complexing with an antibody, or biotin.

The mRNA may be detected by quantitative polymerase chain
reaction.
30

A screening method which comprises separately contacting
each of a plurality of substantially identical samples,
each sample containing a predefined number of cells under
conditions such that contacting is affected with a
35   predetermined amount of each different molecule to be

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tested, is als included.

The plurality of samples may comprises more that about 10^4
samples, or more than about 5 X 10^4 samples.

5

Pursuant to the provisions of the Budapest Treaty on the
International Recognition of Deposit of Microorganisms
for Purpose of Patent Procedure, the plasmid and the
cell lines listed below have been deposited with the
10   American Type Culture Collection ("ATCC"), 12301 Parklawn
Drive, Rockville, Maryland 20852, U.S.A.,:

        1.   a plasmid designated pUV106, deposited under
              ATCC Accession No. 40946.

15

        2.   a human colon adenocarcinoma cell line,
              transfected with pHRA521, designated H21,
              deposited under ATCC Accession No. CRL 10640.

20   3.   a HTB-30 human colon adenocarcinoma cell line,
              transfected with pNEU106, designated N-2,
              deposited under ATCC Accession No. CRL 10658.

        4.   a SW 480 human breast carcinoma cell line,
25   transfected with pKRAS106, designated K-2,
              deposited under ATCC Accession No. CRL 10662.

        5.   a K562 cell line, transfected with pNEU106,
              designated bK108, deposited under ATCC
30   Accession No. CRL 10665.

        6.   a NIH Swiss mouse embryo cell line, NIH 3T3,
              transfected with the MMTV reporter plasmid,
              designated M10, deposited under ATCC Accession
35   No. CRL 10659.

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Further provided is a m thod f essentially simultaneously screening molecules to determine whether the molecules are capable of transcriptionally modulating
5   one or more oncogenes or tumor suppressor genes according to the methods described above.

A screening method where more than about 10^3 samples per week are contacted with different molecules is
10   encompassed by this invention.

Further provided is a method for directly transcriptionally modulating in a multicellular organism the expression of an oncogene or a tumor suppressor gene,
15   the expression of which is associated with a defined physiological or pathological effect in the organism. This method comprises administering to the organism an a molecule at a concentration effective to transcriptionally modulate expression of the gene and
20   thus affect the defined physiological or pathological effect.   In this method the molecule (a) does not naturally occur in the organism, (b) specifically transcriptionally modulates expression of the oncogene or the tumor suppressor gene, and (c) binds to DNA or RNA,
25   or binds to a protein at a site on such protein which is not a ligand-binding domain of a receptor which naturally occurs in the cell, the binding of a ligand to which ligand-binding domain is normally associated with a defined physiological or pathological effect.
30
The molecule may bind to a modulatable transcription sequence of the gene. The molecule may comprise an antisense nucleic acid, a double-stranded nucleic acid molecule, a nucleic acid capable of forming a triple
35   helix with d uble-stranded DNA.   The multicellular

33

organism may b an animal including a human being.

The physiological effect may be the pr t ction of non-
neoplastic cells from damage by chemotherapeutic agents
5   and the defined pathological effect may be a disorder and
modulated expression of an oncogene or tumor suppressor
gene is associated with amelioration of the disorder.
The defined pathological effect may be cancer or
leukemia. The cancer may include malignant melanoma,
10 lung cancer, colon cancer, pancreatic cancer, ovarian
cancer or breast cancer.

The administering in the preceeding method may comprise
topical contact, oral, transdermal, intravenous,
15 intramuscular or subcutaneous administration.

Methods of administration of molecules in the practice of
the invention are well known to those skilled in the art
as are methods of formulating the molecule for
20 administration depending on the specific route of
administration being employed.

This invention is illustrated in the Experimental Detail
section which follow. These sections are set forth to
25 aid in an understanding of the invention but are not
intended to, and should not be construed to, limit in any
way the invention as set forth in the claims which follow
thereafter.

EXPERIMENTAL DETAILS

MATERIALS AND METHODS

5   A. Cell Culture

All media and reagents used for routine cell culture were
purchased from Gibco (Grand Island, NY), Hazelton
(Lenexa, KS), or Whittaker M.A. Biologicals
10   (Walkersville, MD). Fetal calf serum (FCS) was from
Hyclone (Logan, UT).

A human colon adenocarcinoma cell line, SW480 (ATCC CCL
228) was used for experiments concerning expression of
15   the three ras proto-oncogenes and the p53 tumor
suppressor gene. This cell line was maintained on DMEM,
15% fetal calf serum (FCS), 1% Nonessential amino acids
(NEAA). Stable transfectants of this cell line were
selected in the same medium with the addition of G418
20   (Geneticin, Gibco) to a final concentration of 0.6 mg/ml.

A human breast adenocarcinoma derived cell line, SK-BR-3
(ATCC HTB 30) was used for the experiments concerning
expression of the neu (erbB2) proto-oncogene. This cell
25   line was maintained on DMEM, 15% FCS and 1 μg/ml insulin.
Stable transfectants of this cell line were selected in
this same medium with the addition of G418 to a final
concentration of 0.4 mg/ml.

30   A human chronic myelogenous leukemia derived cell line,
K562 (ATCC CCL 243) was used for experiments concerning
the expression of the phl promoter.

A human hepatocellular carcinoma derived cell line, Hep3B
35   (ATCC# HB8064), was used for transfection of plasmids

containing the cyt megal virus (CMV) promoter (us d as a
c ntr l f r th high throughput screen). Thes cells
were maintained on MEM:OptiMEM (1:1) supplemented with
10% FCS.

5

A murine embryonic fibroblast cell line, NIH3T3 (ATCC#
CCL92), was used for the transfection of plasmids
carrying the MMTV promoter (a control cell line). These
cells were maintained on DMEM, supplemented with 10% FCS.

10

B. Construction of the Luciferase-Fusion Reporter Vector

Unless otherwise indicated, molecular cloning procedures
were performed essentially according to Maniatis et al.
15 (42).   Oligonucleotides   were   synthesized   by   the
beta-cyanoethyl phosphoramidite method according to
protocols   provided   by   the   manufacturer   of   the
DNA-synthesizer (Model 380A, Applied Biosystems (Foster
City, CA).

20

A mammalian expression shuttle vector was designed to
allow the construction of the promoter-reporter gene
fusions to be used in high-throughput screens to identify
transcriptionally modulating chemicals. Features of the
25 plasmid are shown in Figure 1. The shuttle vector was
constructed in several steps.

The firefly luciferase gene was removed from the plant
expression plasmid pDO432 (111) (Figure 2) as a 1.9 kb
30 BamHI fragment and cloned into the BamHI site of pSVL
(Pharmacia, Piscataway, NJ), a mammalian expression
vector containing the SV40 promoter. The resulting
plasmid (pSVLuci; Figure 3) was digested with XhoI and
SalI to produce a 2.4 kb fragment containing the
35 luciferase coding sequences and the SV40 late

36

p lyadenylation sit . This fragment was inserted into
the XhoI site of pMSG (Pharmacia, Piscataway, NJ), a
eucaryotic   expression   vector   containing   the   MMTV
promoter. The resulting MMTV promoter-luciferase fusion
5   plasmid (pMLuci; Figure 4) was used to transfect NIH/3T3
cells as described below. Similar constructs can be made
using luciferase vectors from Clontech (Palo Alto, CA).

Six   oligonucleotides   (pUV-1   through   pUV-6)   were
10   synthesized (see Figure 5 for sequence)(SEQ ID NO: 1-6).
The sequences of pUV-1, pUV-2 and pUV-3 correspond to a
multicloning site, the beta-globin leader sequence and
the   first   53   bases   of   the   firefly   luciferase   coding
region.   The sequences of pUV-4, pUV-5 and pUV-6 are
15   complementary to the first three oligonucleotides. The
pUV oligonucleotides were annealed, ligated and inserted
into   the   SalI/EcoRI   sites   of   pTZ18R   (Pharmacia,
Piscataway NJ) (Figure 6). The resulting vector was then
digested   with   SmaI/PvuII   and   the   oligonucleotide
20   containing fragment was cloned into the pBluescriptKS(+)
plasmid (Stratagene, La Jolla, CA), previously digested
with   PvuII,   to   yield   pUV001   (Figure   6).   Several
fragments were ligated into pUV001 to create pUV100. The
luciferase coding sequences (except first 53 bases) and
25   polyadenylation   site   were   obtained   as   a   1.8   kilobase
XbaI/XmaI fragment from pMLuci (section B-1, Figure 4).
The   SV40   early   splice   site   and   the   SV40   late
polyadenylation   site   were   obtained   as   an   871   bp
XmaI/BamHI fragment from pMSG (Pharmacia, Piscataway NJ,
30   Figure 7). Both DNA fragments were cloned into pUV001,
previously digested with XbaI/BamHI to yield pUV100
(Figure 7).

A   476   bp   fragment   c ntaining   a   dimeric   SV40
35   polyad nylati n site was then cloned into the BclI site

f pUV100 (Figure 8). To do this, a 238 bp BclI/BamHI
fragm nt was btained fr m SV40 gen mic DNA (BRL),
ligated, digested with BclI/BamHI, gel is lated, and
inserted into pUV100, resulting in the vector pUV100-3

5   (Figure 8). Linkers containing one SfiI and one NotI
restriction site were then cloned into the PvuII/BamHI
sites of pUV100-3. Two sets of linkers were synthesized
containing the SfiI site in opposite orientations
(oligonucleotides   D-link1   and   D-link2   and

10   oligonucleotides R-link1 and R-link2). The sequences of
the oligonucleotides (SEQ ID NO: 7-10) were:

        5' GATCGGCCCCTAGGGCCGCGGCCGCAT 3' (D-link1)
        5' ATGCGGCCGCGGCCCTAGGGGCC 3' (D-link2)

15   5' GATCGGCCCTAGGGGCGGCCGCAT 3' (R-link1)
        5' ATGCGGCCGCGGCCCCCTAGGGCC 3' (R-link2)

The plasmid that contains D-link oligonucleotides was
named pUV102 and the plasmid that contains R-link

20   oligonucleotides was named pUV103 (Figure 9).

The neomycin resistance gene (neo) was then placed under
control of the Herpes Simplex Virus thymidine kinase
(HSV-TK) promoter to generate a resistance cassette which

25   is free of known enhancer sequences. To do this the
HSV-TK   promoter   was   synthesized   using   four
oligonucleotides (Figure 10) (SEQ ID NO: 11-14) designed
according to published sequence information (44), and
including an SfiI restriction site 5' of the HSV-TK

30   sequences. These oligonucleotides were phosphorylated,
annealed, ligated and inserted into pUV100 digested
previously with HindIII/NheI, generating the vector pTKL
100 (Figure 11). After verifying the HSV-TK sequence,
the about 3.5 kb NheI/SmaI fragment was isolated from

35   pTKL100, and the about 0.9 kb BstBI/BglII fragment

containing the n o coding region was isolated from
pRSVNEO (45). These tw fragm nts wer filled in with
Klenow polymerase and ligated to form pTKNEO (Figure 12).
An additional SfiI site was then inserted 3' of the neo
5   gene by isolating the about 1.8 kb SfiI/BamHI and about
2.6 kb SfiI/PVUII fragments of pTKNEO and conducting a
three way ligation along with a synthesized SfiI
oligonucleotide generating pTKNEO2 (Figure 13)(SEQ ID NO:
15-16). The HSV-TK/NEO vector containing an optimized
10   Kozac sequence was also utilized (Stratagene, La Jolla,
CA, pMC1NEO). An additional vector was constructed by
replacing the about 0.9 kb EcoRI/SalI fragment of pTKNEO2
with the about 0.9 kb EcoRl/SalI fragment from pMC1NEO.
This vector was termed pTKNEO3. (Figure 14). The SfiI
15   fragment of pTKNEO3, containing the TK promoter and the
neomycin resistance gene, was cloned into the SfiI site
of pUV102 to yield pUV106.

C. Molecular Cloning of Oncogene and Tumor Suppressor
20   Promoters and Insertion into the Mammalian Expression
Shuttle Vector

Strategy: This section describes (a) the molecular
cloning of the promoter and transcriptionally modulatable
25   regulatory sequences of the human (I) neu (c-erbB2), (II)
K-ras, (III) H-ras, (IV) N-ras proto-oncogenes, the (V)
phl promoter (responsible for the expression of the phl-
abl oncogene fusion following the translocation event
characteristically observed in chronic myelogenous
30   leukemia (generating the Philadelphia chromosome), and
the (VI) p53 tumor suppressor gene, and (b) the making of
constructs where these regulatory sequences control the
expression of the firefly luciferase gene. These
constructs were transfected into cells as described in
35   below and analyzed for their utility as reporters for the

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discovery of gene expression modulating c mpounds (f r example, in a high-throughput scre n to identify chemicals acting as specific transcripti nal modulators.

5   To make such constructs, several kilobases of sequence upstream of the transcription start site, along with 5' untranslated sequences up to the translation start site (ATG), of a gene of interest were inserted 5' of the luciferase coding region, along with any additional

10   sequences (e.g. intronic enhancers) required for properly regulated expression of the luciferase reporter. In this way constructs can be made where all sequences upstream of their translation start site are from the gene of interest, and all coding sequences are from the

15   luciferase gene. How this was accomplished for the individual oncogenes is described below.

1. neu (c-erbB2) reporter vector

20   Oligonucleotide probes based on the published sequence (46) of the 5' region of the c-erbB2 gene were synthesized and used to screen a human leukocyte genomic library (Clontech Inc.). A 3.2 kb BglI fragment from a positive plaque, containing the upstream regulatory

25   elements, the 5' untranslated leader and exon 1 was then subcloned into pBluscriptKS(+), generating pNEU001. A 1.8 kb HincII-NcoI fragment from pNEU001, containing the upstream regulatory elements and most of the 5' untranslated leader was purified by preparative gel

30   electrophoresis and ligated into pUV103 previously digested with SnaBI and NcoI, generating pNEU002. Two oligonucleotides (SEQ ID NO: 17-18) were synthesized:

     5'-CATGGGGCCGGAGCCGCAGTGAGCAC-3' and
35   5'-CATGGTGCTCACTGCGGCTCCGGCCC-3'

40

These oligonucleotides were annealed to on another,
ph sph rylated and ligated into NcoI digested pNEU002,
generating pNEU103. The synth tic linker fuses the DNA
coding for the _neu_ 5' untranslated leader to the
5   luciferase open reading frame such that the AUG utilized
for translation initiation of the _neu_ gene forms the
first codon of the luciferase gene. The ScaI-XbaI
fragment of pNEU103, containing vector sequences, the
upstream regulatory elements, the 5'untranslated leader
10 and a portion of the luciferase open reading frame, was
purified by preparative gel electrophoresis and ligated
into pUV106 which had previously been digested with ScaI
and XbaI, generating pNEU106 (Figure 15). Linearized
pNEU106 was used in the transfections to generate the
15 _neu_-luciferase reporter cell lines as described below.

2. K-ras reporter vector

Oligonucleotides based on the published K-_ras_ sequence
20 (47) were used to isolate two genomic clones by standard
methods from a human leukocyte library (Clontech). DNA
from these two phages was subcloned into pBluscriptKS(+)
(Stratagene) generating pKS4 and pKS11.

25 A 4 kb XhoI-StuI fragment of pKS11, containing most of
intron 1 and exon 1 up to a point 11 bases 5' of the
point of translation initiation, was isolated by
preparative gel electrophoresis and ligated into XhoI-
StuI digested pGEM7Zf (Promega) which had been previously
30 modified by inserting an adaptor the ApaI and XhoI sites
in the original vector. This adaptor comprised of two
oligonucleotides (5'-TCGAGATCTGAGGCCTGCTGACCATGGGGGGCC-3'
and 5'-CCCATGGTCAGCAGGCCTCAGATC-3') (SEQ ID NO: 19-20)
annealed to one another and was used to allow the proper
35 alignment of the K-_ras_ ATG initiator codon with the

41

luciferase ORF in the final construct (b low).   The
resulting plasmid was designated pGEM715.

A 3 kb HinDIII-XhoI fragment from pKS4, comprising 2.2 kb
5   of K-ras untranscribed upstream DNA and sequences coding
for exon 0 and part of intron 1 was purified by
preparative gel electrophoresis and ligated into pGEM715
which ad been previously digested with HinDIII and XhoI
to generate pGEM7.
10

A 7.7 kb HinDIII-NcoI fragment of pGEM7, comprising 2.2
kb of K-ras upstream regulatory elements, exon 0, intron
1, and part of exon 1 (to the ATG at the NcoI site), was
purified by preparative gel electrophoresis and ligated
15   int pUV102 which had previously been digested with
HinDIII and NcoI to generate pKRAS102.   The TK-Neo
fragment from pTKNeo3 was then ligated into the SfiI site
of pKRAS102 to generate pKRAS106 (Figure 16), the vector
used foe transfections to generated the stable reporter
20   cell lines.

3.   H-ras reporter vector

A 6.4 kb BamHI fragment, containing the entire H-ras gene
25   was isolated from a genomic DNA library by conventional
means. This BamHI fragment was subcloned into the BamHI
site of pSV2Neo (48) generating pHRAS001.   A 1.7 kb
BamHI-MstII fragment containing the H-ras untranscribed
regulatory elements and the 5' untranslated leader
30   including intron-1 (up to within 9 bp of the sequences
encoding the H-ras ATG translation initiation site) was
purified by preparative gel electrophoresis, and along
with an adaptor (5'-TGAGGAGC-3' and 5'-CATGGCTCC-3'
annealed together) ligated into BglII-NcoI cut pUV102
35   generating pHRAS102. The TK-Neo3 fragment of pTKNeo3 was

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then ligat d int th SfiI site f pHRAS102 t generate
pHRAS106 (Figure 17), the v ct r used to generat the
stable H-ras reporter cell line.

5   4. N-ras reporter vector

Oligonucleotides, based on the published sequence of the
human N-ras gene (57) are synthesized and used to screen
a human leukocyte genomic library (Clontech). Positive
10   plaques are subcloned as 8 kb EcoRI fragments containing
the upstream regulatory elements, 5' untranslated leader,
exons 1 and 2, intron 1 and a portion of intron 2, into
pBluscriptKS(+), generating pNRAS001. A 4.5 Kb NcoI-
HinDIII fragment containing the upstream regulatory
15   elements and most of the 5' untranslated leader, is
purified by preparative gel electrophoresis and ligated
along with a 75 bp synthetic HinDIII-NcoI linker, into
pUV102 which had previously been digested with NcoI,
generating pNRAS102. The SfiI pTKNeo3 fragment
20   containing the neomycin resistance gene was then ligated
into the SfiI site of pNRAS102, generating, pNRAS106 the
final reporter vector (Figure 18).

5. phl-abl reporter vector

25

For the isolation of the phl promoter, five
oligonucleotides were synthesized which were
complementary to regions within the first exon. These
oligonucleotides were used to screen a human leukocyte
30   genomic library (Clontech). Positive clones were
restriction mapped, subcloned and sequenced. One
subclone contained 534 bases upstream of the putative
transcription start site (based on a published cDNA
sequence), the region corresponding to the long 5'
35   untranslated leader (472 bases), and 175 bases of the

coding regi n in th first x n (Figure 19).   This
subcl ne was used t construct a series f f ur promoter-
luciferas report r vectors, each with a different amount
of 5' upstream DNA. These plasmids are illustrated in
5   Figure 20. Plasmid 1 consists of the 4 kb Xba I fragment
ligated into Xba I-Spe I digested pUV103. Plasmid 2
consists of the 1.2 kb Bgl II-Xba I fragment ligated into
Bgl II-Xba I digested pUV103. Plasmid 3 consists of Bal
I-SnaB I deletion of Plasmid 2. Plasmid 4 consists of
10 the 135 bp Xma I-Xba I fragment inserted into pUV103.

6. p53 reporter vector

The arrangement of promoters, 5' untranslated exons and
15 a large (10 kb) first intron precludes the construction
of a p53 reporter vector which includes all of the
promoter elements and the complete untranslated leader
such that the AUG initiator codon of the normal p53 ORF
becomes the first codon of the luciferase ORF. As an
20 alternative, fusion constructs which are similar to those
successfully employed by others for the analysis of the
expression of this gene are used.   There are two
promoters which play a role in the regulated expression
of the p53 tumor suppressor gene. Because it is not yet
25 clear which of these two promoters is the most important
for determination of the cellular level of the p53
protein, two plasmids are constructed, each fusing one of
the promoters to the luciferase open reading frame.
Oligonucleotides based on the published sequence are used
30 as probes to isolate genomic p53 clones from a human
leukocyte library (Clontech). Subclones of this original
clone serve as a source of the fragments used to
construct both of the p53 reporter vectors. The p53P1
containing region is isolated as a 2.4 kb EcoRI-XbaI
35 fragment essentially as described in reference (49). This

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fragment is rendered blunt end d by Klen w fragment and
ligat d into SnaBI digested pUV106, the proper
rientati n f the fragment is c nfirm d by restriction
mapping, generating pP531106 (Figure 21). The p532
5   promoter is isolated as a 1.4 kb XbaI-BglII fragment,
essentially as described in (50). The XbaI site is
rendered blunt ended with Klenow fragment prior to the
secondary digestion by BglII. The resulting fragment is
purified by preparative gel electrophoresis, and this
10   fragment is then ligated into pUV106 which had previously
been digested with SnaBI and BglII, generating pP532106
(Figure 22).

7.   CMV reporter vector

15

A 580 bp cytomegalovirus genomic fragment containing the
immediate early promoters and enhancers (51) was ligated
into pUV100 previously digested with NotI and NheI and
rendered blunt ended by treatment with Klenow fragment,
20   generating pUVCM. An 888 bp NaeI-XbaI fragment from
pUVCM, including the CMV promoter and enhancers plus a
portion of the luciferase coding region, was purified by
preparative gel electrophoresis and ligated into pUV106
which had previously been digested with SnaBI and XbaI,
25   generating pCM106 (Figure 23), the vector used to
transfect the CMV reporter cell lines.

D.   Construction of Mini-genes

30   As a secondary analysis of compounds identified as able
to repress the synthesis of specific oncogenes, a series
of "mini-genes" were constructed. A mini-gene is the
fusion of promoter elements identical to those used to
construct luciferase reporter vectors, fused to the cDNA
35   of the activated oncogene. These constructs, when

45

transfected int an appr priate h st cell lin , lead to
its phen typic transf rmation. Comp unds which repress
th activity of th r gulat ry elements l ad t a
reversal of the transformed phenotype. The construction
5   of the c-erbB2 and K-ras minigenes are described below.

1.   c-erbB2 minigene

The 1.7 kb erbB2 promoter fragment (SalI-NcoI) was
10   purified from pNEU102 and combined an NcoI-SalI cDNA
fragment isolated form a commercially available breast
carcinoma cDNA library (Clontech) according to the
supplier's directions. A 26 bp linker was inserted into
the NcoI site to correctly fuse the untranslated leader
15   to the ATG initiation codon (Figure 24).

2.   K-ras minigene

The K-ras promoter fragment was isolated as a HinDIII-
20   StuI fragment from pKRAS106. A K-ras cDNA containing
plasmid was obtained from the ATCC (ATCC# 41027) and
mutated by oligonucleotide mutagenesis (Ammersham) to
generate a cDNA encoding a mutant K-ras protein with an
aspartic acid at position 12. A StuI-XhoI fragment
25   containing the K-ras coding region was ligated to the
promoter fragment, generating the final minigene (Figure
25).

E. Liquid Scintillation Counter Bioluminescence Assay
30

To assay for luciferase expression in transient
expression assays in the various transfected clones (see
below), cells were incubated with various transcriptional
inducers in serum free defined media, washed 3 times with
35   Dulbecco's phosphate-buffered saline (D-PBS, Gibco) and

lysed in Lysis Buffer 1 (50 mM Tris acetate pH 7.9, 1 mM EDTA, 10 mM magnesium acetate, 1 mg/ml bovine serum albumin [BSA], 0.5% Brij 58, 2 mM ATP, 100 mM dithiothreitol [DTT]). All reagents were obtained from

5   Sigma except for DTT which was from Boehringer Mannheim. After lysis, cell debris was sedimented by brief centrifugation, and 950 μl of supernatant extract were added to a glass scintillation vial. Samples were counted individually in an LKB (Gaithersburg, MD)

10   scintillation counter on a setting which allows measurement of individual photons by switching off the coincidence circuit. The reaction was started by addition of 50 μl of 2mM luciferin (Sigma, St. Louis, MO or Boehringer. Mannheim, Indianapolis IN) in Buffer B

15   (Buffer B-Lysis Buffer 1 without Brij 58, ATP and DTT) to the 950 μl of lysate. Measurement was started 20 seconds after luciferin addition and continued for 1 minute. Results were normalized to protein concentration using the Bradford protein assay (BioRad, Richmond CA) or to

20   cell numbers using Trypan Blue (Sigma) exclusion counting in a hemocytometer.

F. Transfection

25   Cell were transfected by one of three methods, following manufacturer's instructions; by calcium phosphate precipitation (Pharmacia), lipofection (Life Technologies Inc.) or electroporation (BioRad). In most cases, 25-75 μg of plasmid DNA, linearized by a single restriction

30   endonuclease cut within the vector sequences, was electroporated into approximately 5 million cells. When co-transfection of a separate neomycin resistant plasmid was employed the molar ratio of luciferase fusion plasmid to neomycin resistant plasmid was either 10:1 or 20:1.

35   Neomycin resistant clones were selected by growth in

media c ntaining G418 (Geneticin, Gibco).

G. Southern blotting

5   To monitor correct and complete stable integration of
     transfected   promoter/reporter   constructs,   stably
     transfected cell clones were subjected to Southern blot
     analysis (52). Genomic DNA was prepared of each clone to
     be tested and restriction-cut with DraI or another
10   appropriate   restriction   endonuclease.   After
     electrophoresis,   transfer   to   nylon   filters   and
     immobilization by UV irradiation using a Stratalinker UV
     device (Stratagene, La Jolla, California), integrated
     promoter/luciferase fusion constructs were visualized by
15   probing with radioactively labelled XbaI-EcoRI fragments
     of the luciferase coding region. Probes were labelled
     using the random primer method (53). Since DraI cuts in
     the SV40 polyadenylation sites located in the mammalian
     expression shuttle vector just upstream the inserted
20   promoter   sequences   as   well   as   downstream   of   the
     luciferase coding region, but not in most of the
     promoter sequences used for generating stably transfected
     cell clones, a single fragment should be visualized by
     the probe used. The size of that fragment should be
25   characteristic   for   each   of   the   promoter   sequences
     analyzed.

H. Isolation of Total Cellular RNA

30   Total cellular RNA was isolated from the luciferase-
     fusion containing cell clones or from untransfected host
     cells following incubation with various transcriptionally
     modulating chemicals known previously to affect gene
     expression. Total cellular RNA was isolated using the
35   RNAZol   method   (CINNA/BIOTECX,   Friendswood,   TX,

48

Lab rat ries Internati nal, Inc.). Cells were
r suspended and lys d with RNAZol solution (1.5 ml/9 cm
petri dish) and the RNA was solubilized by passing the
lysate a few times through a pipette. Chloroform was
5   added to the homogenate (0.1 ml/1.0 ml), and samples were
shaken for 15 seconds followed by a 5 minute incubation
on ice. After centrifuging for 10 minutes, the upper
phase was collected and an equal volume of isopropanol
was added. Samples were incubated for 45 minutes at
10   -20°C, and the RNA was pelleted for 15 minutes at 12,000
x g at 4°C. The RNA pellet was then washed with 70%
ethanol and dried briefly under vacuum.

I.   Polymerase Chain Reaction

15

Total RNA was isolated using the approach described
above, first strand cDNA generated by either oligo dT,
random hexamer or gene specific oligonucleotide priming.
Specific amplification oligonucleotides were added, and
20   the polymerase chain reaction carried out according to
established methods (54).

RNA levels are quantitated by established methods (55)
which include the addition of varying amounts of a
25   control RNA and thereby establishing a standard curve.
PCR products are visualized on an ethidium bromide
stained agarose gel and are quantitated by measuring the
incorporation of radiolabelled deoxynucleotide
triphosphates using liquid scintillation.

30

J.   Specific PCR mRNA Detection and Quantitation of
Oncogenes and Tumor Suppressor Genes

35   Oligonucleotides (SEQ ID NO: 21-34) were designed for the

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specific d t cti n f each f the gen s t b analyzed:

K-<u>ras</u>:   5'-CTCTTGGATATTCTCGACACACAGCA-3'
                5'-GTGTCTACTGTTCTAGAAGG-3'

H-<u>ras</u>:   5'GCACGCACTGTGGAATCTCGG-3'
                5'-ACGTCGAGTACGTCGGCCCCGGTG-3'

N-<u>ras</u>:   5'-GCCCACGAACTGGCCAAGAGTT-3'
                5'-CTGAGTCCCATCATCACTGCTCTT-3'

<u>neu</u>:   5'-CCAATGCCAGCCTGTCCTTCCTGCAG-3'
                5'-GATCAAGACCCCTCCTTTCAAGATCT-3'

<u>phl-abl</u>:   5'-ATTCCGCTGACCATCAATAAGGAAG-3'
                5'-ATAGCCTAAGACCCGGAGCTTTTCAC-3'

p53:   5'-GATGGAGAATATTTCACCCTTCAG-3'
                5'-CTGACCCTTTTTGGACTTCAGGTGG-3'

M-CSF   5'-CTCCAGCCCGCAGCTCCAGGAGTCTG-3'
                5'-CCCTCTACACTGGCAGTTCCACCTG-3'

In each case, these oligonucleotides were chosen to amplify sequences which span intron splice junctions in order to minimalize the nonspecific signal generated by contaminating genomic DNA (55). In the case of the K-<u>ras</u> oligos bind to sequences within exons II and III, flanking intron 2. In the case of H-<u>ras</u> and N-<u>ras</u>, the oligos bind to sequences within exons III and IV, flanking intron 3. In the case of c-<u>erb</u>B2, the oligos bind to sequences in exons II and IV flanking introns 2 and 3 and amplifying DNA corresponding to exon III. In the case of <u>phl-abl</u> the oligos bind to sequences within exon III of the <u>bcr</u> region and exon II of <u>abl</u> flanking

50

tw introns in the chimeric message and amplifying DNA
c rr sponding to ex n I f abl. In the case f p53 the
oligos bind to sequences within exons IX and XI and
amplify DNA corresponding to exon X.   The
5   oligonucleotides for M-CSF are used in control
experiments and are complementary to regions of M-CSF
exons VI and VIII and amplify the region corresponding to
exon VII.

10   K. High Throughput Quantitative PCR

Current methods for measuring changes in gene expression
suffer from various limitations. Conventional direct
analysis of changes in mRNA levels (nuclease protection,
15 Northern blot, primer extension) lack sufficient
sensitivity for use with high throughput formats (e.g. 96
well plate cell culture). These methods also require
difficult analytical procedures (e.g. sequencing gels)
complicating automation.   The use of gene fusions
20 (luciferase or CAT transcriptional fusions) as
demonstrated above, provide sufficient sensitivity and
ease of analysis but require disruption of the native
transcription unit and loss of chromosomal context,
leading to potential artifact.   This section of the
25 invention proposes to circumvent the sensitivity
limitation of direct analysis by using the amplification
potential inherent in the polymerase chain reaction.
Combining PCR with the ease of florescence detection of
will allow direct mRNA analysis in a high throughput
30 mode.

The following description outlines a high throughput drug
screen utilizing direct PCR quantitation of mRNA in its
most simple format.

35

1.   <u>Grow cells</u>. Cells are grown in 96 well micr titer
     plates as d scrib d above. The final detection step
     is a flu rescenc measurement, so an paque (non-
     reflecting black) plate is required.

2.   <u>Add compounds</u>. As with the luciferase reporter
     screen, compounds are added at several
     concentrations and at several replications. The
     number of duplicate samples required can be
     determined statistically after the basic assay is
     formatted (currently, quadruplicates are required).

3.   <u>Incubate</u>. The incubation time depends on the
     biology of the systems studied. As with the current
     luciferase reporter assay, the incubation time is 24
     hours.

4.   <u>Lyse cells</u>: The cells are lysed in a buffer which
     satisfies several important criteria: A. avoidance
     of extremes of temperature; B. complete inactivation
     of contaminating cellular nucleases; C. compatible
     with subsequent RNA purification steps; D. rapid
     and efficient lysis. Chaotropic buffers have been
     described which satisfy these requirements.
     Guanidine HCl (6M) will efficiently lyse cells and
     effectively inactivate cellular nucleases. The
     kinetics of nucleic acid hybridization are largely
     unaffected by these conditions. Thus, the
     subsequent RNA purification (separation using
     magnetic oligo dT beads, see below) does not require
     a buffer change.

5.   <u>Add external control</u>: The exponential nature of the
     PCR amplification step tends to magnify small
     differences in conditions. Key to the usefulness of

52

this approach is the car ful inclusi n f appr priate standards f r c ntrol purposes. An artificial polyadenylated RNA (generated with phage T7 RNA polymerase and commercially available vectors, Promega Madison WI) is added at this point. This RNA serves as a control for all of the following steps: purification, cDNA synthesis, PCR amplification, PCR product purification and detection. This control RNA is added to several lysates at varying concentrations, generating a standard curve.

6. <u>Purify RNA</u>: RNA is purified using commercially available oligo-dT tagged magnetic beads. These beads are added to the lysate, allowed to hybridize to the mRNA, brought to the bottom of the plate using a strong magnet, and extensively washed to remove protein and DNA.

7. <u>Synthesize cDNA</u>: cDNA is generated using the 3' end of the bead bound oligo-dT.

8. <u>Add PCR primers</u>: Each gene (or control) to be assayed requires two oligos. The pair are designed so that they span a large intron. This makes the amplification much more RNA specific. The short, spliced RNA target is much more efficiently amplified than the longer, contaminating genomic DNA target. One of the oligo pair is tagged at its 5' end with a fluorescent label. The other oligo is tagged at its 5' end with biotin (for future purification, see below). Several sets of oligos are added to each lysate. A set for each control and a set for each gene to be assayed. Every set has a different fluorescent tag.

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9. <u>PCR Amplification</u>: Simultane us incubati n of many
    plates is frequently r quir d, s   ither a large
    array f bl cks or a larg capacity convection ov n
    is necessary.

10. <u>Purification of PCR products</u>: The PCR products are
    separated from the unreacted oligos using a method
    similar to the one employed for the initial RNA
    purification.   Magnetic beads, tagged with
    streptavidan are added to the mixture. PCR products
    are tagged on one end with biotin (on the other with
    a fluorescent label) and tightly attach to the
    magnetic beads. The beads, along with the labeled
    oligonucleotides, are brought to the bottom of the
    plate with a magnet and extensively washed.
    Alternatively, fluorescently tagged PCR products are
    resolved electrophoretically and quantitated with a
    scanning gel fluorimeter (Applied Biosystems).

11. <u>Detection</u>:   The plates are read in a 96 well
    fluorimeter (Ammersham).

12. <u>Data analysis</u>:   A ratio of fluorescence from a
    particular gene's PCR product to the signal from the
    "constitutive" internal control gives the relative
    mRNA level. Changes in this ratio indicates a
    change in gene expression. Absolute mRNA levels are
    determined by control experiments using carefully
    quantitated artificial RNAs to construct standard
    curves for each gene studied. This establishes a
    given ratio (to the internal control) for a given
    cellular RNA concentration.

L.   Isolation of Single Cell Clones Containing Various Promoter-Luciferase Fusion Constructs

1. pMluci into NIH3T3 (MMTV control cell line)

pMluci and pSV2Neo, an antibiotic resistance plasmid (48), were co-transfected into NIH/3T3 mouse fibroblast cells using the calcium phosphate precipitation method (56) with a commercially available kit (Pharmacia, Piscataway NJ). Two days later, cells were transferred to media containing 0.4 mg/ml G418 and were grown for an additional 10-14 days. G418-resistant clones were isolated by standard methods. Once sufficient cell numbers were obtained, clones were analyzed based on several criteria: constitutive luciferase production, induction of luciferase expression by dexamethasone (1 μm, Sigma, St. Louis, MO), satisfactory attachment to microtiter plates used in the high-throughput screen (see section G) and acceptable standard deviation in multiple luciferase expression assays (see below for assay protocol). This analysis was carried out using the luciferase assay conditions described above. Of the clones which satisfied the above criteria for the high throughput screen, one clone, M10, was selected for use.

2. neu (pNEU106) into HTB30

75 micrograms of the pNEU106 plasmid was linearized by a single restriction endonuclease cleavage within the vector backbone and electroporated into HTB30 human breast carcinoma cells. Neomycin resistant clones were isolated and tested for luciferase activity. Clones testing positive for luciferase production were subjected to Southern blot analysis (see below). The best clone (producing the highest signal and carrying a single

55

intact c py of the transf cted DNA) was utilized for high
throughput scr ning (designat d cl ne N-2).

3. K-ras (pKRAS106) into SW480

75 micrograms of the pKRAS106 plasmid was linearized by
a single restriction endonuclease cleavage within the
vector backbone and electroporated into SW480 human
breast carcinoma cells. Neomycin resistant clones were
isolated and tested for luciferase activity. Clones
testing positive for luciferase production were subjected
to Southern blot analysis (see below). The best clone
(producing the highest signal and carrying a single
intact copy of the transfected DNA) was utilized for high
throughput screening (designated clone K-2).

4. H-ras (pHRA521) into human colon adenocarcinoma cell line

75 micrograms of the pHRA521 plasmid was linearized by a
single restriction endonuclease cleavage within the
vector backbone and electroporated into a human colon
adenocarcinoma cell line. Neomycin resistant clones were
isolated and tested for luciferase activity. Clones
testing positive for luciferase production were subjected
to Southern blot analysis (see below). The best clone
(producing the highest signal and carrying a single
intact copy of the transfected DNA) was utilized for high
throughput screening (designated clone H21)

6. phl (pPHL106) into K562

75 micrograms of the pPHL106 plasmid was linearized by a
single restriction endonuclease cleavage within the
vector backbone and electroporated into K562 cells.

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Neomycin resistant clones were isolated and test d for
luciferase activity. Clones testing positive for
luciferase production were subjected to Southern blot
analysis (see below). The best clone (producing the
highest signal and carrying a single intact copy of the
transfected DNA) was utilized for high throughput
screening (designated clone bK108).

7. p53 (pP531106 and pP532106) in SW480

75 micrograms of both the pP531106 and pP532106 plasmids
are linearized by a single restriction endonuclease
cleavage within the vector backbone and separately
electroporated into SW480 human colon carcinoma cells.
Neomycin resistant clones are isolated and tested for
luciferase activity. Clones which test positive for
luciferase production are subjected to Southern blot
analysis (see below). The best clones (producing the
highest signal and carrying a single intact copy of the
transfected DNA) representing each plasmid are utilized
for high throughput screening (designated clones P531 and
P532).

8. pCM106 into Hep3B (a control cell line)

Hep3B hepatocellular carcinoma cells were transfected by
electroporation with 75 micrograms of pCM106 which had
been linearized by a single ScaI cut within the vector
backbone. Neomycin resistant colonies were isolated and
tested for luciferase activity. Luciferase positive,
neomycin resistant clones were subjected to Southern blot
analysis (see below). The best clone, producing the most
luciferase activity from a single, correctly integrated
vector was selected for use as the CMV reporter cell line
in the HTP screen (this clone was designated CM1).

57

M. High-Throughput (HTP) Screening

Cell plating: Dynatech Micr liter 96 well plates were
custom pretreated for cell attachment by Dynatech
Laboratories, Inc. (Chantilly, VA). Alternatively, the
96 well plates were treated with 50 μl per well of human
fibronectin (hFN, 15 μg/ml in PBS, Collaborative
Research, Bedford, MA) overnight at 37°C. hFN-treated
plates were washed with PBS using an Ultrawash 2
Microplate Washer (Dynatech Labs), to remove excess hFN
prior to cell plating. N-2 (the neu reporter) K-2 (the
K-ras reporter) and CM1 (the CMV reporter, added as a
control) maintained in their respective serum media (with
0.2 mg/ml G418) were washed with PBS, harvested by
trypsinization, and counted using a hemocytometer and the
Trypan Blue exclusion method according to protocols
provided by Sigma, St. Louis, MO Chemical Company. Cells
were then diluted into media (with 0.2 mg/ml G418), and
0.2 ml of cell suspension per well was plated using a
Millipore MultiDrop Dispenser (Millipore Corp., Bedford,
MA).   Plates were incubated overnight at 37°C in a
humidified 5% CO_2 atmosphere. Chemicals from the chemical
file of one of Oncogene Science's corporate partners were
dissolved in DMSO at concentrations of 3-30 mg/ml. The
next day a fully automated device, as described in U.S.
patent application #382,483, was used to incubate
luciferase reporter cells in 96-well microtiter plates,
transfer chemicals and known transcriptional modulators
to the cells, incubate cells with the chemicals, remove
the chemicals by washing with PBS, add lysis buffer to
the cells and measure the bioluminescence produced. The
cell lysis buffer was modified to also contain the
luciferin.   Therefore, lysis of cells and the
bioluminescence reaction begin simultaneously and the
production of bioluminescent light reaches a maximum at

58

ab ut 5 min. The level of light output declines by about 20% within furth r 30 min. For better lysis buffer stability bovine serum albumin has been omitted. This improved lysis buffer has been shown to remain fully functional for at least 12 hours, when kept on ice and protected from direct light.

59

RESULTS

A. In vivo signal half-life of the luciferase reporter system

When screening for inhibitors rather than inducers of transcription, the half-life of the reporter molecule becomes a crucial parameter in determining the minimal incubation time that would be necessary to allow enough decay of reporter molecules so that the inhibition of their synthesis became visible. The oncogene reporter cell line were therefore tested for the time dependency of luciferase activity after treatment of the cells with Actinomycin D, an inhibitor of transcription. This experiment measured the combined half-life of luciferase mRNA and of the luciferase protein and compares the rate of signal decay of the H-ras, K-ras and c-erbB2 reporter cell lines to a CMV reporter cell line control. Cells derived from clones CM1 (CMV), K-2 (K-ras), H21 (H-ras) and N-2 (c-erbB2) were seeded into 96-well microtiter plates and incubated overnight. At time 0, Actinomycin D (25 µg/ml) was added. At the times indicated in Figure 26, cells were washed with PBS and luciferase activity of Actinomycin-treated cells determined as described in Materials and Methods. The signal from the treated cells was compared to the luciferase activity of untreated controls. The logarithm of the treated/untreated ratio was plotted versus time, this data is shown in Figure 26. The calculated half-life of the signal from each of the four cell lines is shown in table 1. The half-lives were found to range from about 3 to 10 hours indicating that a 24 hour incubation with a 100% efficient inhibitor of transcription would be sufficient to reduce luciferase levels to 6% of the control in the tested cell lines.

60

TABLE 1
Half-life Determinations

Cell Line	Reporter	Signal Half-life
CM1	CMV	6.5 hours
K2	K-ras	6.5 hours
H21	H-ras	10 hours
N2	neu	3 hours

B.   Southern Blot

An autoradiogram from a typical Southern blot analysis is
shown in Figure 27.   Genomic DNA from five neomycin
5   resistant, luciferase producing clones, transfected with
the H-ras reporter vector pHRAS106 was digested with
DraI, blotted and probed as described in Materials and
Methods, along with two control samples; one consisting
only of genomic DNA from the untransfected host strain
10   (SW480) and the other consisting of a mixture of SW480
genomic DNA and DraI digested pHRAS106 (to provide a size
control for the expected band.   In this case all five
clone produced the expected band (and no other)
demonstrating that the transfected vector had integrated
15   into the host cell genome with out undergoing gross
rearrangement.   In other transfections, correctly
integrated vectors represented between 100% and 20% of
the neomycin resistant, luciferase producing clones.

20   **C. PCR Detection and Quantitation**

To test the quantitative ability of the polymerase chain
reaction, total RNA was isolated form U5637 bladder
carcinoma cells and diluted in two-fold serial steps to

yi ld samples ranging from 4 to 0.05 µg. Th se RNA
sampl s w re us d to g nerat cDNA using rand m primers
(55) and then mixed with a c nstant amount of phage
lambda DNA (0.2 ng) as a control for amplification
5   efficiency.   Alpha-32P-dATP was included in the PCR
buffer to allow quantitation of the amplified products.
The M-CSF specific PCR oligonucleotides describe above
were added to the standard reaction mixture (2 pmoles per
sample) and PCR carried out for 35 cycles in a Perkin-
10   Elmer-Cetus thermal cycler. The products of the reaction
were electrophoresed on a 3% NuSieve agarose gel. The
gel was dried and used to expose Kodak X-OMAT AR film.
The resulting autoradiogram is shown in Figure 28. This
autoradiogram was quantitated using an LKB laser
15   densitometer, the data are shown in Figure 29. The graph
plots the amount of M-CSF specific product divided by the
constant lambda DNA signal. The reaction was clearly
quantitative for the RNA samples between 0.05 and 1 µg
(total RNA), and proved to be a very sensitive assay for
20   M-CSF mRNA, which is barely detectable in this cell line
by conventional S1 analysis.

To test the specificity of the PCR reaction, the oligos
for detection of c-erbB2, N-ras, H-ras and K-ras were
25   used to amplify cDNA derived from 10 and 0.4 ng of U5637
total RNA. The data are shown in Figure 30. In each
sample a sample of phage lambda DNA and lambda control
oligos was added to control for the efficiency of the PCR
reaction. This is the uppermost, consistent band in each
30   lane. In each case the oligos specifically amplifies a
DNA fragment of the correct molecular weight. A few
extra bands of unknown origin appear in the c-erbB2 lanes
and in the lane representing the highest concentration of
H-ras mRNA.

35

D. Quality Assurance Analysis

A number of quality assurance criteria are routinely
assessed during the course of high throughput screens.
5   Data from QA analysis of a portion of Screen III are
shown in Figures 31-34. Figure 31 shows an analysis of
the consistency of the luciferase signal on various areas
of each plate. The ratios of negative control values
from three different areas within each plate are
10 calculated and plotted versus plate number. The expected
value is 1.0. Values greater than 1.5 or less than 0.4
indicate uneven signal generation across the plate. In
this example 240 plates, representing 1440 compounds,
tested against three cell lines, are shown. The
15 coefficient of variance for the 12 negative control
values from each of the same 240 plates are represented
by the data shown in Figure 32. Values less than 20% are
considered acceptable. Similar data for the 12 positive
control values of the same plates are shown in Figure 33.
20 Figure 34 shows the transcription induction ratio (TIR)
for the positive controls of one cell line represented in
the same set of 240 plates. The TIR is the ratio of the
experimental values to the untreated controls. In this
case the cell line is the K-ras reporter and the positive
25 control is Actinomycin D a potent general inhibitor of
transcription. Three values are shown for each plot,
representing three different concentrations of
Actinomycin D. The expected value for such an analysis
depends on the half life of the signal and the incubation
30 time (here 24 hours), but for this combination, typical
values range from 0.4 to 0.3 fold.

E. High-Throughput Drug Screen

       Table 2 presents the data from a thr e week high
throughput screen of 2334 compounds. Three cell lines
were utilized; CM1 (the CMV reporter cell line) as a
control for nonspecific effects. N-2 (the c-erbB2
reporter cell line) and K-2 (the K-ras reporter cell
line). Each compound was assayed at three concentrations
in quadruplicate. Each microtiter plate included a
negative control row (no added compound) and a positive
control row (Actinomycin D at three concentrations). The
data are reported as TIR (transcription induction ratio)
which is the median of the samples quadruplicate values
divided by the median of the negative control values. In
this case transcriptional inhibitors are sought, so the
selection criteria for lead compounds is that the test
promoter be inhibited to 0.4 of the negative control
while the other cell lines remain within 0.8X of the
control value. During these three weeks 10 compounds
scored positive for the specific inhibition of the K-ras
promoter, 19 scored as leads for the inhibition of the c-
erbB2 promoter and 39 compounds inhibited
nonspecifically. Compounds scoring as leads in the
primary screen are repeated and then subjected to
secondary analysis such as effects on the minigene
transfectant phenotypes (see above).

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     Gene Transcription</u>. (C hen, P. and F ulkes, G.J.,
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

    (i) APPLICANT: Oncogene, Science Inc.

    (ii) TITLE OF INVENTION: Methods of Transcriptionally
Modulating Expression of Oncogenes and Tumor Suppressor
10 Genes

    (iii) NUMBER OF SEQUENCES: 34

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20   (F) ZIP: 10112

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
25   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version
#1.25

    (vi) CURRENT APPLICATION DATA:
30   (A) APPLICATION NUMBER:
        (B) FILING DATE: 17-JAN-1992
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45 (2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 23 base pairs
        (B) TYPE: nucleic acid
50   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

     <u>5</u>   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGATCGCAG CGCTGCCTTT CCT
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<u>10</u>   (2) INFORMATION FOR SEQ ID NO:2:

     (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 31 base pairs
        (B) TYPE: nucleic acid
<u>15</u>   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

<u>20</u>

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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        (D) TOPOLOGY: linear

<u>35</u>   (ii) MOLECULE TYPE: DNA (genomic)

<u>40</u>   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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   (2) INFORMATION FOR SEQ ID NO:4:
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        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
<u>50</u>   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

5   CGCTATGGAG TTGGCTCAAG CAGCCTGC
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    (2) INFORMATION FOR SEQ ID NO:5:

10   (i) SEQUENCE CHARACTERISTICS:
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                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear
15
            (ii) MOLECULE TYPE: DNA (genomic)

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    GGCGGGTCTG TAGGCAGGTC GGCTC
                    25

25 (2) INFORMATION FOR SEQ ID NO:6:

            (i) SEQUENCE CHARACTERISTICS:
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                (B) TYPE: nucleic acid
30   (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)

35

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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    (2) INFORMATION FOR SEQ ID NO:7:

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                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

50   (ii) MOLECULE TYPE: DNA (genomic)

71

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTCCAGCCCG CAGCTCCAGG AGTCTG
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5

(2) INFORMATION FOR SEQ ID NO:8:

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10   (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)
15

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

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            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
30
    (ii) MOLECULE TYPE: DNA (genomic)

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCCAAGGAG GCCGAGAATA TCACG
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40 (2) INFORMATION FOR SEQ ID NO:10:

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            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
45   (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

50

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

72

GCCAGACTTC TACGGCCTGC TGCCCGAC
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(2) INFORMATION FOR SEQ ID NO:11:

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    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCAGCAATTG AGAGCATTCT TAAA
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(2) INFORMATION FOR SEQ ID NO:12:

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    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(2) INFORMATION FOR SEQ ID NO:13:

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    (A) LENGTH: 30 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACTAATAATG TAAAAGACGT CACTAAATTG

5

10

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    (2) INFORMATION FOR SEQ ID NO:14:

<u>5</u>   (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 25 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

<u>10</u>
       (ii) MOLECULE TYPE: DNA (genomic)

<u>15</u>   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

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              25

<u>20</u> (2) INFORMATION FOR SEQ ID NO:15:

        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 40 base pairs
           (B) TYPE: nucleic acid
<u>25</u>   (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

<u>30</u>

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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<u>35</u>   40

    (2) INFORMATION FOR SEQ ID NO:16:

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<u>40</u>   (A) LENGTH: 26 base pairs
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

<u>45</u>   (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
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CATGGGGCCG GAGCCGCAGT GAGCAC
         26

(2) INFORMATION FOR SEQ ID NO:17:

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        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATGGTGCTC ACTGCGGCTC CGGCCC
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        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TCGAGATCTG AGGCCTGCTG ACCATGGGGG CC
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(2) INFORMATION FOR SEQ ID NO:19:

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        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

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               (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: DNA (genomic)

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     CTCTTGGATA TTCTCGACAC ACAGCA
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     (2) INFORMATION FOR SEQ ID NO:21:

          (i) SEQUENCE CHARACTERISTICS:
               (A) LENGTH: 20 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: DNA (genomic)

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

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     (2) INFORMATION FOR SEQ ID NO:22:

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               (A) LENGTH: 21 base pairs
               (B) TYPE: nucleic acid
               (C) STRANDEDNESS: single
               (D) TOPOLOGY: linear

          (ii) MOLECULE TYPE: DNA (genomic)

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

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                    21

     (2) INFORMATION FOR SEQ ID NO:23:

          (i) SEQUENCE CHARACTERISTICS:

76

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                    (A) LENGTH: 24 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: singl
                    (D) TOPOLOGY: linear
5
            (ii) MOLECULE TYPE: DNA (genomic)


10          (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

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15   (2) INFORMATION FOR SEQ ID NO:24:

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                    (B) TYPE: nucleic acid
20                  (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)

25

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            (i) SEQUENCE CHARACTERISTICS:
35                  (A) LENGTH: 24 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

40          (ii) MOLECULE TYPE: DNA (genomic)


            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
45
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     (2) INFORMATION FOR SEQ ID NO:26:
50
            (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 26 base pairs
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                (B)  TYPE: nucleic acid
                (C)  STRANDEDNESS: single
                (D)  TOPOLOGY: linear

  5        (ii) MOLECULE TYPE: DNA (genomic)


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 10
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                          26

        (2) INFORMATION FOR SEQ ID NO:27:
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                     (A)  LENGTH: 26 base pairs
                     (B)  TYPE: nucleic acid
                     (C)  STRANDEDNESS: single
 20                  (D)  TOPOLOGY: linear

           (ii) MOLECULE TYPE: DNA (genomic)


 25
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 30

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                     (D)  TOPOLOGY: linear

 40        (ii) MOLECULE TYPE: DNA (genomic)


           (xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
 45
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                          26

        (2) INFORMATION FOR SEQ ID NO:29:
 50
                (i)  SEQUENCE CHARACTERISTICS:
                     (A)  LENGTH: 26 base pairs
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                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: singl
                    (D) TOPOLOGY: lin ar

5   (ii) MOLECULE TYPE: DNA (genomic)

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:
10
      ATAGCCTAAG ACCCGGAGCT TTTCAC
                      26

      (2) INFORMATION FOR SEQ ID NO:30:
15
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                    (A) LENGTH: 24 base pairs
                    (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
20   (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)

25
            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

      GATGGAGAAT ATTTCACCCT TCAG
                      24
30
      (2) INFORMATION FOR SEQ ID NO:31:

              (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 25 base pairs
35   (B) TYPE: nucleic acid
                    (C) STRANDEDNESS: single
                    (D) TOPOLOGY: linear

            (ii) MOLECULE TYPE: DNA (genomic)
40

            (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

45   CTGACCCTTT TTGGACTTCA GGTGG
                      25

      (2) INFORMATION FOR SEQ ID NO:32:

50   (i) SEQUENCE CHARACTERISTICS:
                    (A) LENGTH: 26 base pairs
                    (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

5

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

10   CTCCAGCCCG CAGCTCCAGG AGTCTG
                  26

    (2) INFORMATION FOR SEQ ID NO:33:

15   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 26 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear
20

    (ii) MOLECULE TYPE: DNA (genomic)

25   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTCCAGCCCG CAGCTCCAGG AGTCTG
            26

30   (2) INFORMATION FOR SEQ ID NO:34:

      (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
35   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

40

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

CCCTCTACAC TGGCAGTTCC ACCTG
45   25

What is claimed is:

1.   A method of directly transcriptionally modulating
        the expression of an oncogene or tumor suppressor
        gene, the expression of which is associated with a
        defined physiological or pathological effect within
        a multicellular organism, which comprises contacting
        a cell, which is capable of expressing the gene,
        with a molecule at a concentration effective to
        transcriptionally modulate expression of the gene
        and thereby affect the level of the oncogene or
        tumor suppressor gene product(s) encoded by the gene
        which is expressed by the cell, which molecule (a)
        does   not   naturally   occur   in   the   cell,   (b)
        specifically transcriptionally modulates expression
        of the oncogene or tumor suppressor gene, and (c)
        binds to DNA or RNA, or binds to a protein at a site
        on such protein which is not a ligand-binding domain
        of a receptor which naturally occurs in the cell,
        the binding of a ligand to which ligand-binding
        domain is normally associated with a defined
        physiological or pathological effect.

2.   A method of claim 1, wherein the molecule does not
        naturally occur in any cell of a lower eucaryotic
        organism.

3.   A method of claim 1, wherein the molecule does not
        naturally occur in any cell.

4.   A method of claim 1, wherein the molecule is not a
        naturally occurring molecule.

5.   A method of claim 1, wherein the cell is a cell of

the multic llular organism.

6. A method f claim 1, wher in the cell is an animal
    cell.

7. A method of claim 6, wherein the animal cell is a
    human cell.

8. A method of claim 1, wherein the transcriptional
    modulation comprises upregulation of expression of
    the oncogene or tumor suppressor gene.

9. A method of claim 1, wherein the transcriptional
    modulation comprises downregulation of expression of
    the oncogene or tumor suppressor gene.

10. A method of claim 1, wherein the molecule binds to
    a modulatable transcription sequence of the gene.

11. A method of claims 1, wherein the molecule comprises
    an antisense nucleic acid.

12. A method of claim 1, wherein the molecule comprises
    double-stranded nucleic acid.

13. A method of claim 1 wherein the molecule comprises
    a nucleic acid capable of forming a triple helix
    with double-stranded DNA.

14. A method of claim 1, wherein the gene is an
    oncogene.

15. A method of claim 14, wherein the oncogene is a c-
    neu gene.

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16. A m thod of claim 14, wherein the oncogen is a c-K-
    ras gen .

17. A method of claim 14, wherein the oncogene is a c-H-
    ras gene.

18. A method of claim 14, wherein the oncogene is a c-N-
    ras gene.

19. A method of claim 14, wherein the oncogene is a phl-
    abl gene fusion.

20. A method of claim 14, wherein the oncogene is a myc
    gene.

21. A method of claim 14, wherein the oncogene is a fms
    gene.

22. A method of claim 14, wherein the oncogene is a erbA
    gene.

23. A method of claim 14, wherein the oncogene is a p53
    gene.

24. A method of claim 14, wherein the oncogene is a
    retroviral oncogene.

25. A method of claim 1, wherein the gene is a tumor
    suppressor gene.

26. A method of claim 25 wherein the tumor suppressor
    gene is a p53 gene.

27. A method of claim 25 wherein the tumor suppressor
    gene is a retinoblastoma gene.

28. A method f claim 25 wherein the tumor suppressor
     gene is a Wilms' tum r gene.

29. A method of claim 25 wherein the tumor suppressor
     gene is a neurofibromatosis type 1 gene.

30. A method of claim 25 wherein the tumor suppressor
     gene is a DCC gene.

31. A method of claim 25 wherein the tumor suppressor
     gene is an _erbA_ gene.

32. A method of claim 25 wherein the tumor suppressor
     gene is an adenomatous polyposis coli gene.

33. A method of determining whether a molecule not
     previously known to be a modulator of protein
     biosynthesis is capable of transcriptionally
     modulating the expression of an oncogene or tumor
     suppressor gene, which comprises contacting a sample
     which contains a predefined number of cells with a
     predetermined amount of a molecule to be tested,
     each such cell comprising DNA consisting essentially
     of (i) a modulatable transcriptional regulatory
     sequence of the oncogene or tumor suppressor gene,
     (ii) a promoter of the oncogene or tumor suppressor
     gene, and (iii) a DNA sequence encoding a
     polypeptide other than the oncogene or tumor
     suppressor, which polypeptide being capable of
     producing a detectable signal, which DNA sequence is
     coupled to, and under the control of, the promoter,
     under conditions such that the molecule, if capable
     of acting as a transcriptional modulator of the
     oncogene or tumor suppressor gene, causes a
     measurable detectable signal to be produced by the

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polypeptide so expressed, quantitativ ly determining
the am unt f th signal produced, comparing the
amount s determined with the amount of produced
signal detected in the absence of any molecule being
tested or upon contacting the sample with any other
molecule, and thereby identifying the molecule as
one which causes a change in the detectable signal
produced by the polypeptide so expressed, and thus
identifying the molecule as a molecule capable of
transcriptionally modulating the expression of the
oncogene or tumor suppressor gene.

34. A method of determining whether a molecule not
previously known to be a modulator of protein
biosynthesis is capable of transcriptionally
modulating the expression of an oncogene or tumor
suppressor gene, which comprises contacting a sample
which contains a predefined number of cells with a
predetermined amount of a molecule to be tested,
each such cell comprising DNA consisting essentially
of (i) a modulatable transcriptional regulatory
sequence of the gene encoding the oncogene or tumor
suppressor, (ii) a promoter of the oncogene or tumor
suppressor gene, and (iii) a reporter gene, which
expresses a polypeptide, coupled to, and under the
control of, the promoter, under conditions such that
the molecule, if capable of acting as a
transcriptional modulator of the oncogene or tumor
suppressor gene, causes a measurable change in the
amount of the polypeptide produced, quantitatively
determining the amount of the polypeptide so
produced, comparing the amount so determined with
the amount of polypeptide produced in the absence of
any molecule being tested or upon contacting the
sample with any other molecule, and thereby

id ntifying th m lecule as   ne which causes a
chang in the amount of the p lypeptide express d,
and thus identifying the molecule as a molecul
capable of transcriptionally modulating the
expression of the oncogene or tumor suppressor gene.

35. A method of determining whether a molecule not
previously known to be a modulator of protein
biosynthesis is capable of transcriptionally
modulating the expression of an oncogene or tumor
suppressor gene, which comprises contacting a sample
which contains a predefined number of cells with a
predetermined amount of a molecule to be tested,
each such cell comprising DNA consisting essentially
of (i) a modulatable transcriptional regulatory
sequence of the oncogene or tumor suppressor gene,
(ii) a promoter of the oncogene or tumor suppressor
gene, and (iii) a DNA sequence transcribable into
mRNA coupled to and under the control of, the
promoter, under conditions such that the molecule,
if capable of acting as a transcriptional modulator
of the oncogene or tumor suppressor gene, causes a
measurable difference in the amount of mRNA
transcribed from the DNA sequence, quantitatively
determining the amount of the mRNA produced,
comparing the amount so determined with the amount
of mRNA detected in the absence of any molecule
being tested or upon contacting the sample with any
other molecule, and thereby identifying the molecule
as one which causes a change in the detectable mRNA
amount of, and thus identifying the molecule as a
molecule capable of transcriptionally modulating the
expression of the oncogene or tumor suppressor gene.

36. A method of claim 33, 34 or 35, wherein the sample

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comprises c lls in mon lay rs.

37. A method of claim 33, 34, r 35, wh rein the sample
     comprises cells in suspension.

38. A method of claim 33, 34 or 35, wherein the cells
     comprise animal cells.

39. A method of claim 38, where the animal cells are
     human cells.

40. A method of claim 33, 34 or 35, wherein the
     predefined number of cells is from about 1 to about
     5×10^5 cells.

41. A method of claim 40, wherein the predefined number
     of cells is from about 2×10^2 to about 5×10^4
     cells.

42. A method of claim 33, 34 or 35, wherein the
     predetermined amount of the molecule to be tested is
     based upon the volume of the sample.

43. A method of claim 33, 34 or 35, wherein the
     predetermined amount is from about 1.0 pM to about
     20 μM.

44. A method of claim 33, 34 or 35, wherein the
     predetermined amount is from about 10 nM to about
     500 μM.

45. A method of claim 33, 34 or 35, wherein the
     contacting is effected from about 1 to about 24
     hours.

46. A m thod of claim 45, wher in the c ntacting is
    ff ct d from ab ut 2 t about 12 hours.

47. A method of claim 33, 34 or 35, wherein the
    contacting is effected with more than one
    predetermined amount of the molecule to be tested.

48. A method of claim 33, 34 or 35, wherein the molecule
    to be tested is a purified molecule.

49. A method of claim 33, 34 or 35, wherein the
    modulatable transcriptional regulatory sequence
    comprises a cloned genomic regulatory sequence.

50. A method of claim 33, 34 or 35, wherein the DNA
    consists essentially of more than one modulatable
    transcriptional regulatory sequence.

51. A method of claim 33 or 34, wherein the DNA sequence
    encoding the polypeptide is inserted downstream of
    the promoter of the oncogene or tumor suppressor
    gene by homologous recombination.

52. A method of claim 33, wherein the polypeptide is a
    luciferase.

53. A method of claim 33, wherein the polypeptide is
    chloramphenicol acetyltransferase.

54. A method of claim 33, wherein the polypeptide is β
    glucuronidase.

55. A method of claim 33, wherein the polypeptide is β
    galactosidase.

56. A m thod of claim 33, wherein the polypeptide is neomycin ph sphotransferase.

57. A method of claim 33, wherein the polypeptide is alkaline phosphatase.

58. A method of claim 33, wherein the polypeptide is guanine xanthine phosphoribosyltransferase.

59. A method of claim 34, wherein the polypeptide is capable of complexing with an antibody.

60. A method of claim 34, wherein the polypeptide is capable of complexing with biotin.

61. A method of claim 35, wherein mRNA is detected by quantitative polymerase chain reaction.

62. A screening method of claim 33, 34, 35 or 39 which comprises separately contacting each of a plurality of substantially identical samples, each sample containing a predefined number of cells under conditions such that contacting is affected with a predetermined amount of each different molecule to be tested.

63. A screening method of claim 62, wherein the plurality of samples comprises more that about 10^4 samples.

64. A screening method of claim 62, wherein the plurality of samples comprises more than about 5 X 10^4 samples.

65. A method of essentially simultaneously screening

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m lecules to determine whether th molecules are
capabl f transcriptionally modulating one r more
oncog nes r tumor suppressor genes which comprises
essentially simultaneously screening the molecules
against the oncogenes or tumor suppressor genes
according to the method of claim 62.

66. A screening method of claim 64 or 65, where more
than about 10^3 samples per week are contacted with
different molecules.

67. A method for directly transcriptionally modulating
in a multicellular organism the expression of a
oncogene or tumor suppressor gene, the expression of
which is associated with a defined physiological or
pathological effect in the organism, which comprises
administering to the organism a molecule at a
concentration effective to transcriptionally
modulate expression of the gene and thus affect the
defined physiological or pathological effect, which
molecule (a) does not naturally occur in the
organism and (b) specifically transcriptionally
modulates expression of the gene encoding an
oncogene or tumor suppressor gene product(s), and
(c) binds to DNA or RNA, or binds to a protein at a
site on such protein which is not a ligand-binding
domain of a receptor which naturally occurs in the
cell, the binding of a ligand to which ligand-
binding domain is normally associated with a defined
physiological or pathological effect.

68. A method of claim 67, wherein the molecule binds to
a modulatable transcription sequence of the gene.

69. A method of claim 67, wherein the molecule comprises

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an antis nse nucleic acid.

70. A method of claim 67, wherein the molecule comprises
a double-stranded nucleic acid molecule.

71. A method of claim 67, wherein the molecule comprises
a nucleic acid capable of forming a triple helix
with double-stranded DNA.

72. A method of claim 67, wherein the multicellular
organism is a human being.

73. A method of claim 67, wherein the multicellular
organism is an animal.

74. A method of claim 72, wherein the physiological
effect is the protection of non-neoplastic cells
from damage by chemotherapeutic agents.

75. A method of claim 72, wherein the defined
pathological effect is a disorder and modulated
expression of an oncogene or tumor suppressor gene
is associated with amelioration of the disorder.

76. A method of claim 72, wherein the defined
pathological effect is cancer.

77. A method of claim 72, wherein the defined
pathological effect is leukemia.

78. A method of claim 72, wherein the defined
pathological effect is malignant melanoma.

79. A method of claim 72, wherein the defined
pathological effect is lung cancer.

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80. A method f claim 72, wh r in the defined path logical eff ct is col n cancer.

81. A method of claim 72, wherein the defined pathological effect is pancreatic cancer.

82. A method of claim 72, wherein the defined pathological effect is ovarian cancer.

83. A method of claim 72, wherein the defined pathological effect is breast cancer.

84. A method of claim 67 or 72, wherein the administering comprises topical contact.

85. A method of claim 67 or 72, wherein the administering comprises oral, transdermal, intravenous, intramuscular or subcutaneous administration.

Figure 1.
Features of the Mammalian Vect r
pUV102 with Inserted TK-NEO Cassette.

Multi Cloning Site

ß-Globin leader

SV40 poly A

Luciferase
Gene

A
m
p
R

Mammalian Expression
Shuttle Vector

SV40
Early
Splice
and
poly A

TK
Promoter

Neomycin Gene

ori

Figure 2.
pDO432.

Figure 3.
pSVLuci.

Luciferase Gene

BamHI
SmaI
XhoI

poly A
SmaI
BamHI
SalI

pSVLuci
6.7 kb

pBR322
ori

AmpR

**Figure 4.
pMLuci.**

Luciferase Gene

Smal

BamHI

BamHI

SV40 poly A

Smal
Xhol
Sall
Smal

Xhol
Sall

BamHI

**pMLuci
10.0 kb**

SV40 ori

SV40 early promoter

MMTV LTR

HindIII

HindIII

E. coli gpt

pBR322
ori

SV40 early
splice

AmpR

SV40 poly A
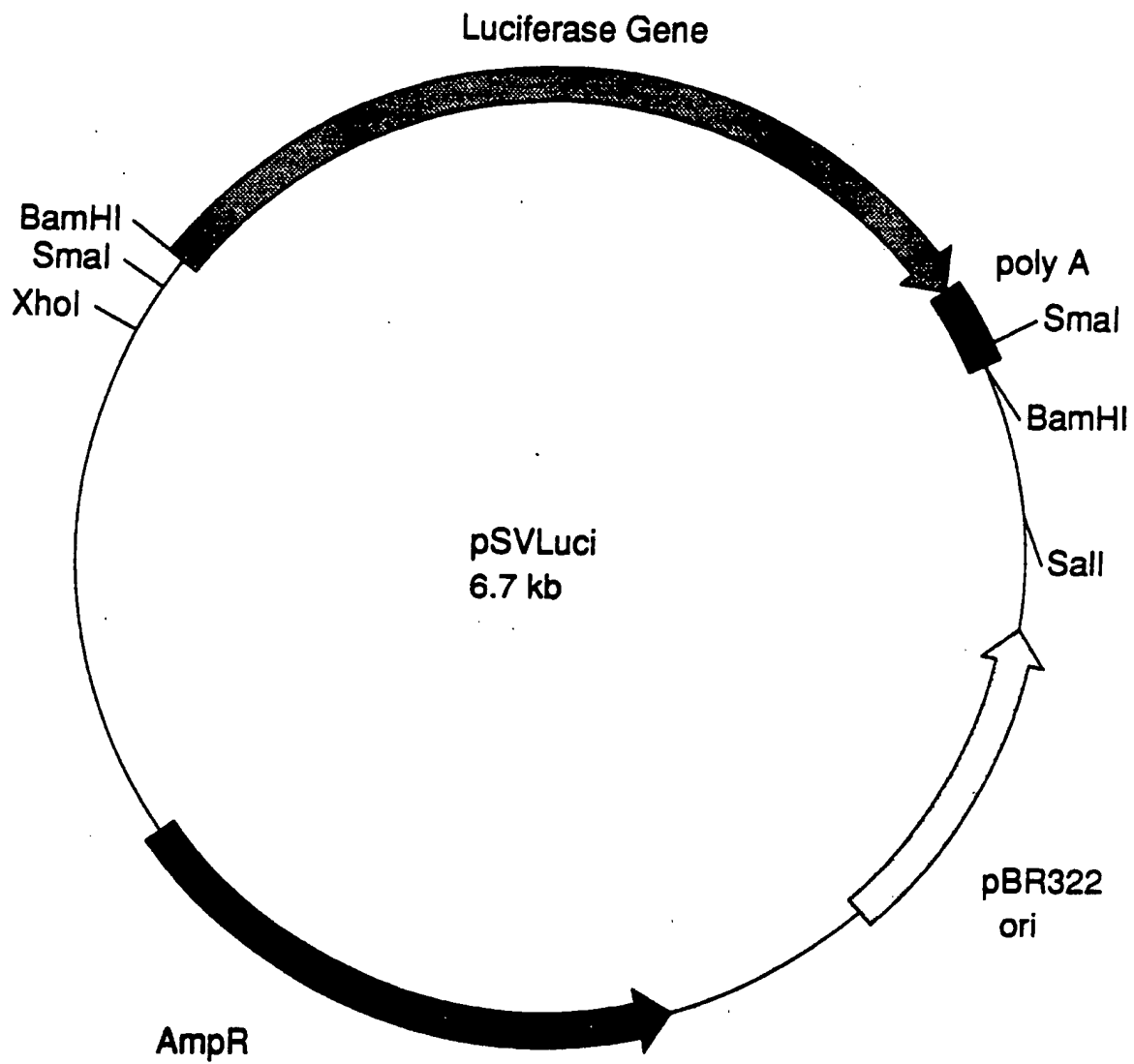
BamHI
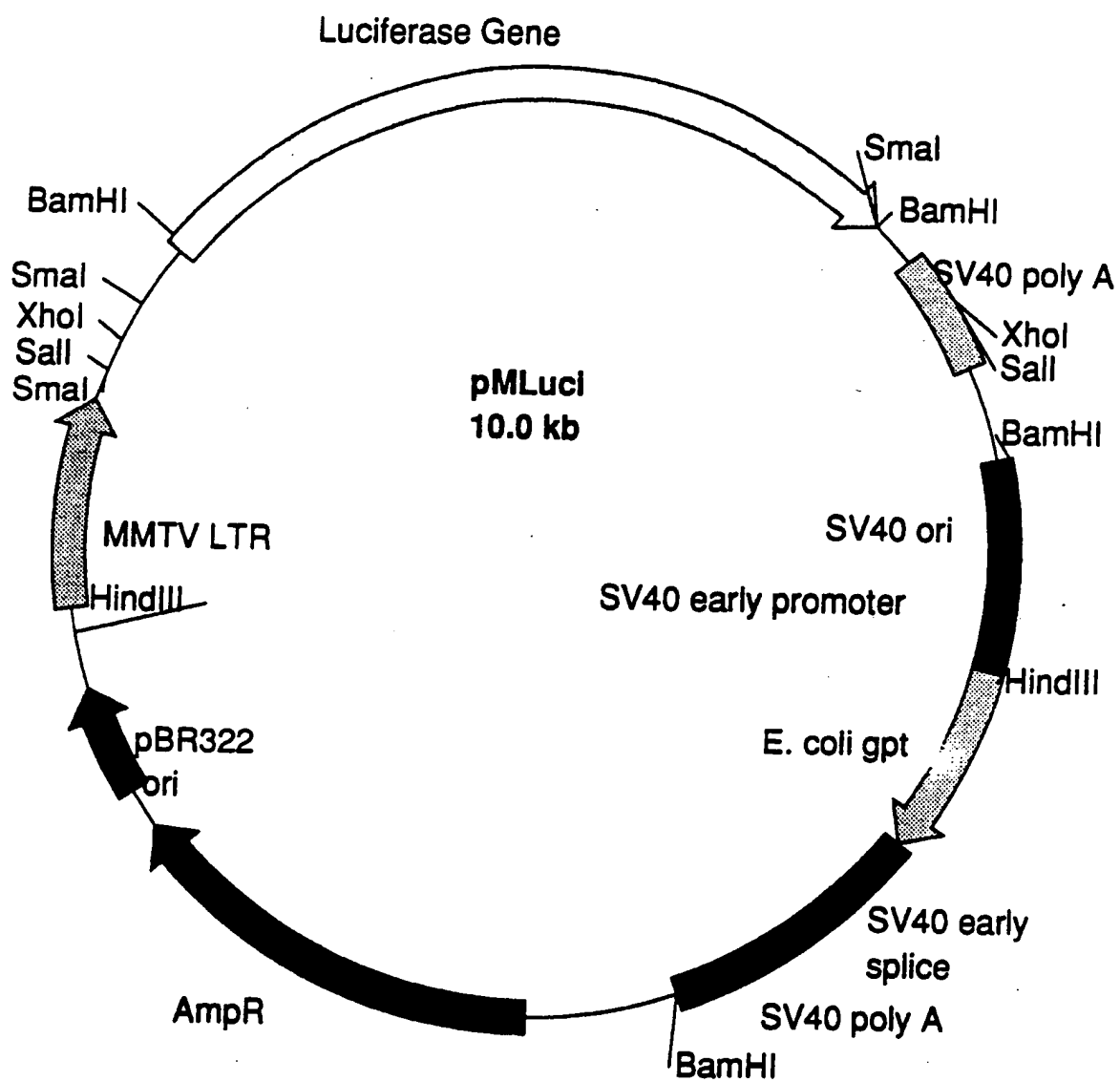
Figure 5.

Sequence of the pUV oligonucleotides.

pUV1:
5'TCGACCCGGGGGCCGCGGCCTGATCAGACGTCGGGCCCGGTACCGGTGCACTACGTAAGATCTAAGCTT3'

pUV2:
5'ACTAGTCTGCAGGCTAGCACTCTTCTGGTCCCCACAGACTCAGAGAGAACCCACCATGGA3'

pUV3:
5'AGACGCCAAAAACATCAAGAAAGGCCCGGCCATTCTATCCTCTAGAGGGGATCCAGCTG3'

pUV4:
5'TAGATCTTACGTAGTGCACGGTACCGGGCCCGACGTCTGATCAGGCGGCCCGGG3'

pUV5:
5'GGTGGGTTCTCTCTGAGTCTGTGGGGACCAGAAGAGTGCTAGCCTGCAGACTAGTAAGCT3'

pUV6:
5'AATTCAGCTGGATCCCCTCTAGAGGATAGAATGGCGGGCCCTTTCTTGATGTTTTTGGCGTCTTCCAT3'

Figure 6

Constructin of pUV001
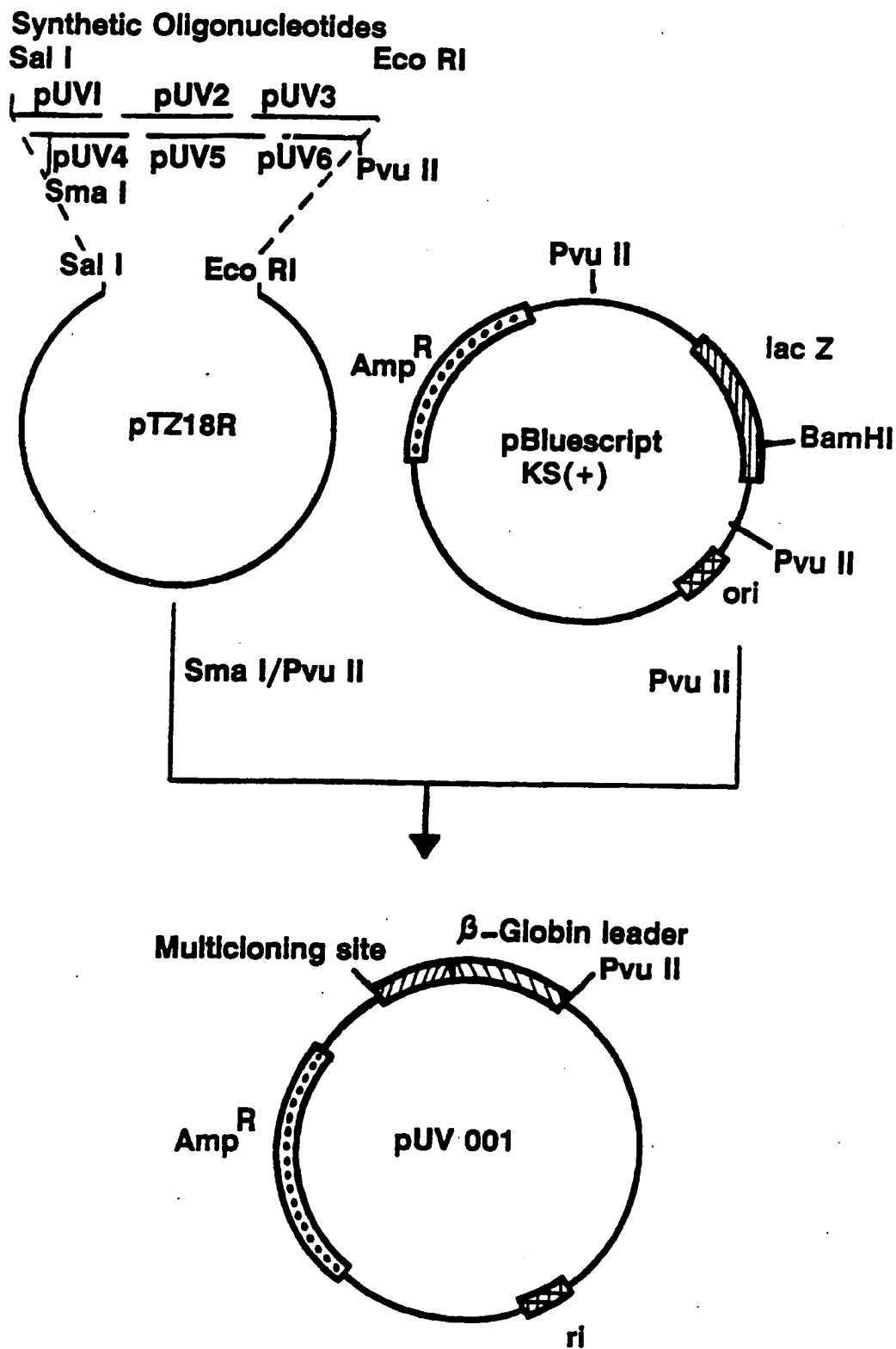
Figure 7
Construction of pUV100
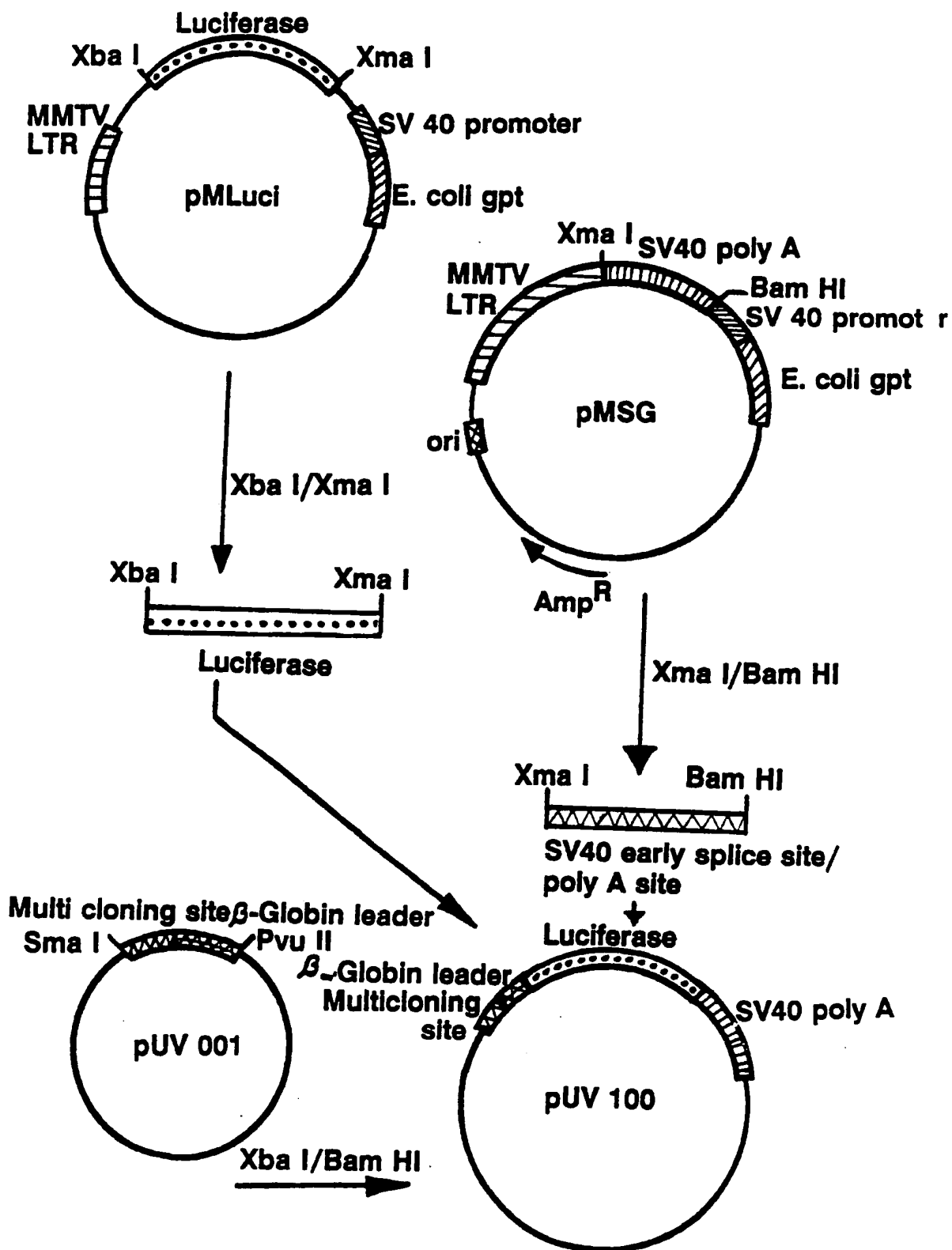
Figure 8
Construction of pUV100-3
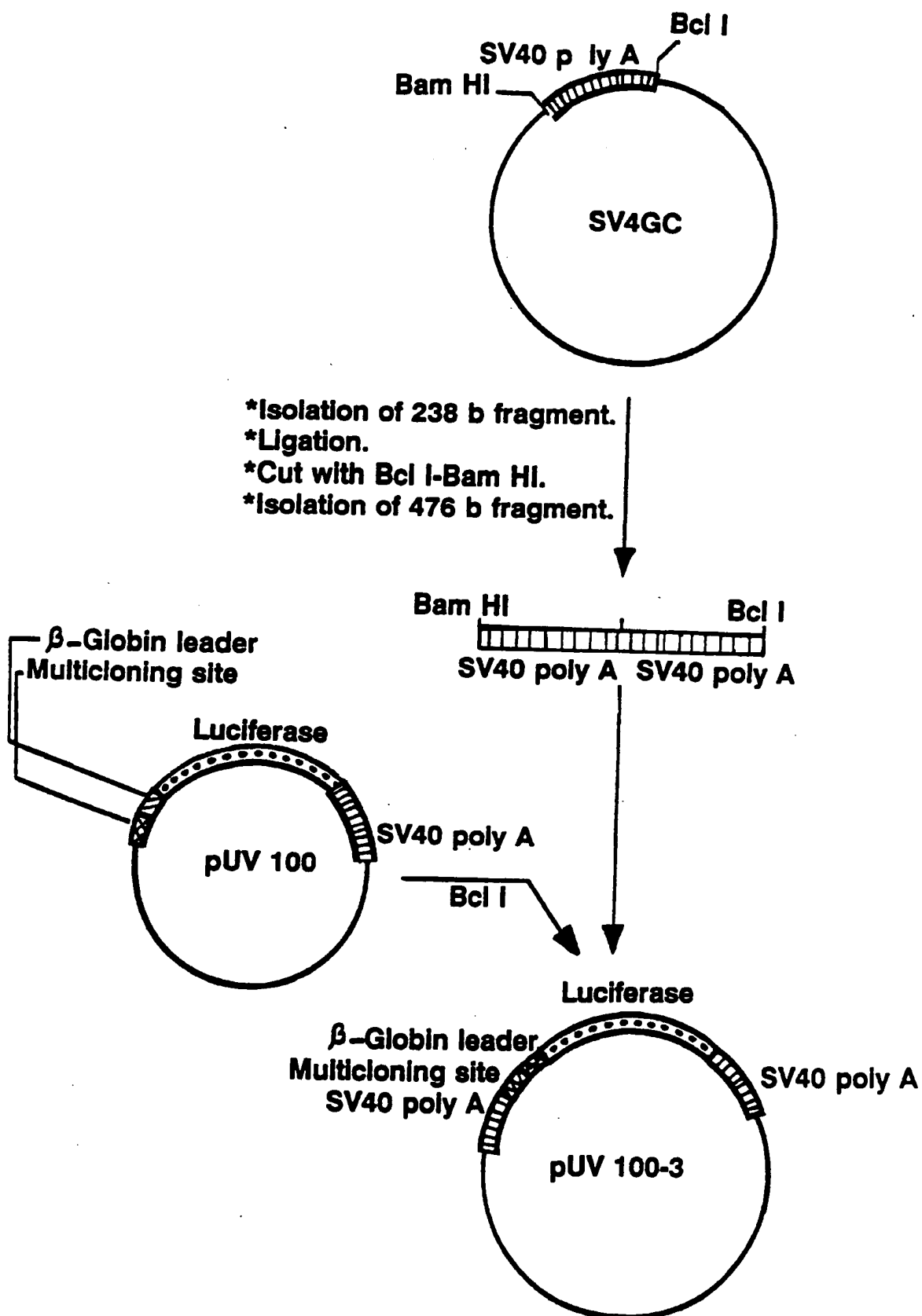
Figur 9

Construction of pUV102 and pUV103
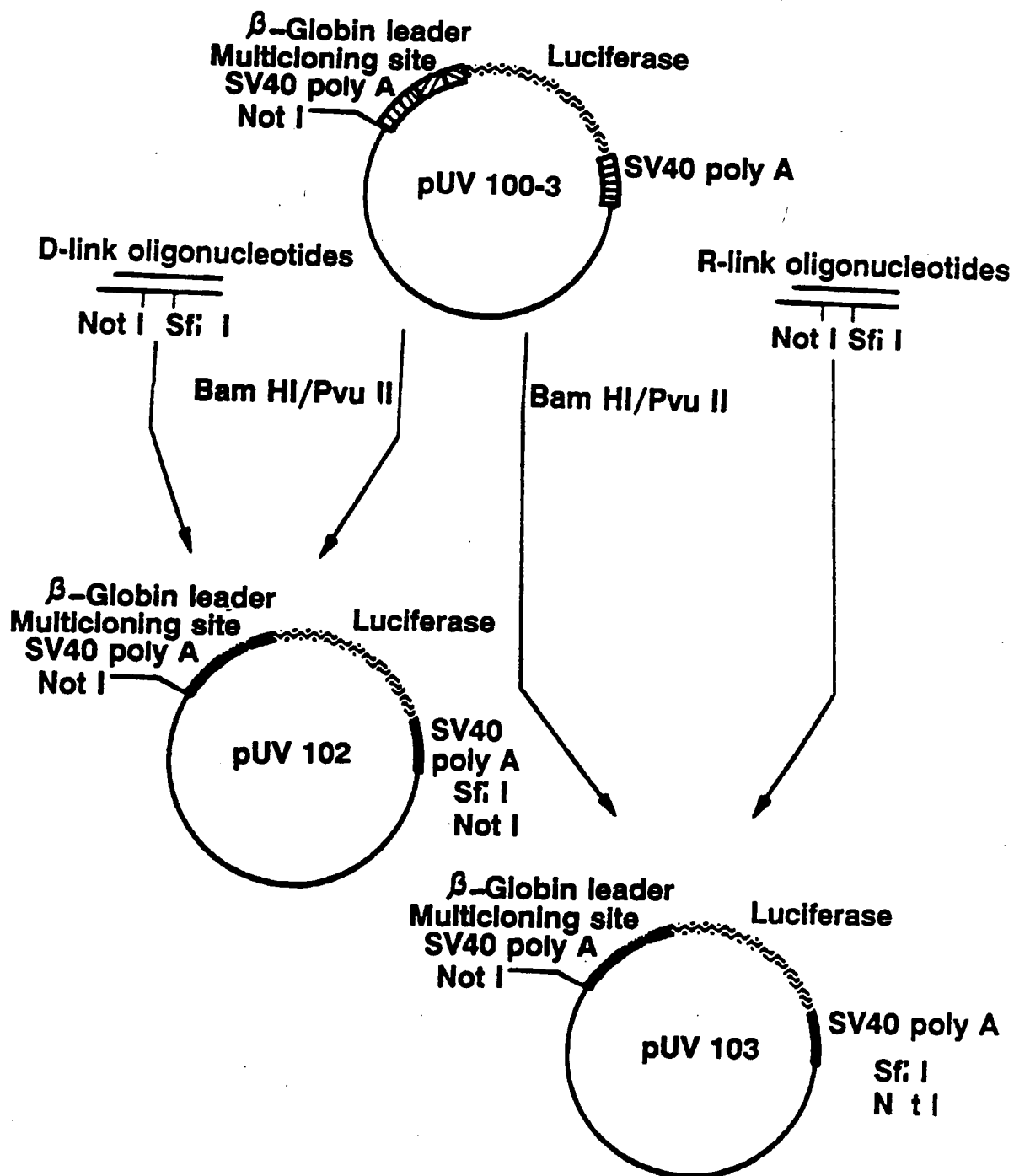
Figure 10

Synthetic HSV-TK Promoter

Oligo #1: 5 AGCTTGGCGCCCCTAGGGCCACTAGTCTGCGAGCTATGATGACACAA
ACCCCGCCCCAGCGTCTTGTCATTGGGCGA-3'

Oligo #2: 3 ACCCGGGGGATCCCGGTGATCAGACTCGATACTACTGTGTTGGGG
CGGGGTCGCGAGAACAGTAAACCGCTTAAGCT-5'

Oligo #3: 5 ATTCGAACACGCAGAGATGCAGTCGGGGGGGGGCGCGGTCCGGAGGTC
CACTTCGCGCATATTAAGGTGACGCGGTGTGGGG-3'

Oligo #4: 3 TGTGCGTCTACGTCAGCCCCGCGCGCGCCAGGCTCCAGGTGAAG
CGTATAATTCCACTGCGCACACCCGATC-5'
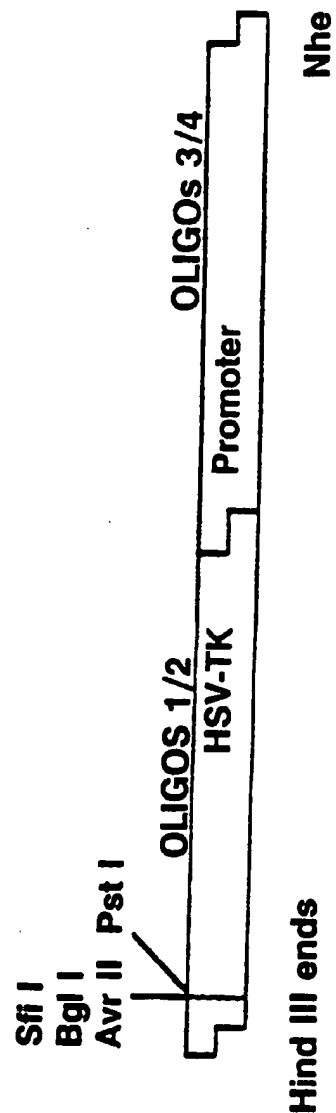
Figure 11

Constuction of pTKL100
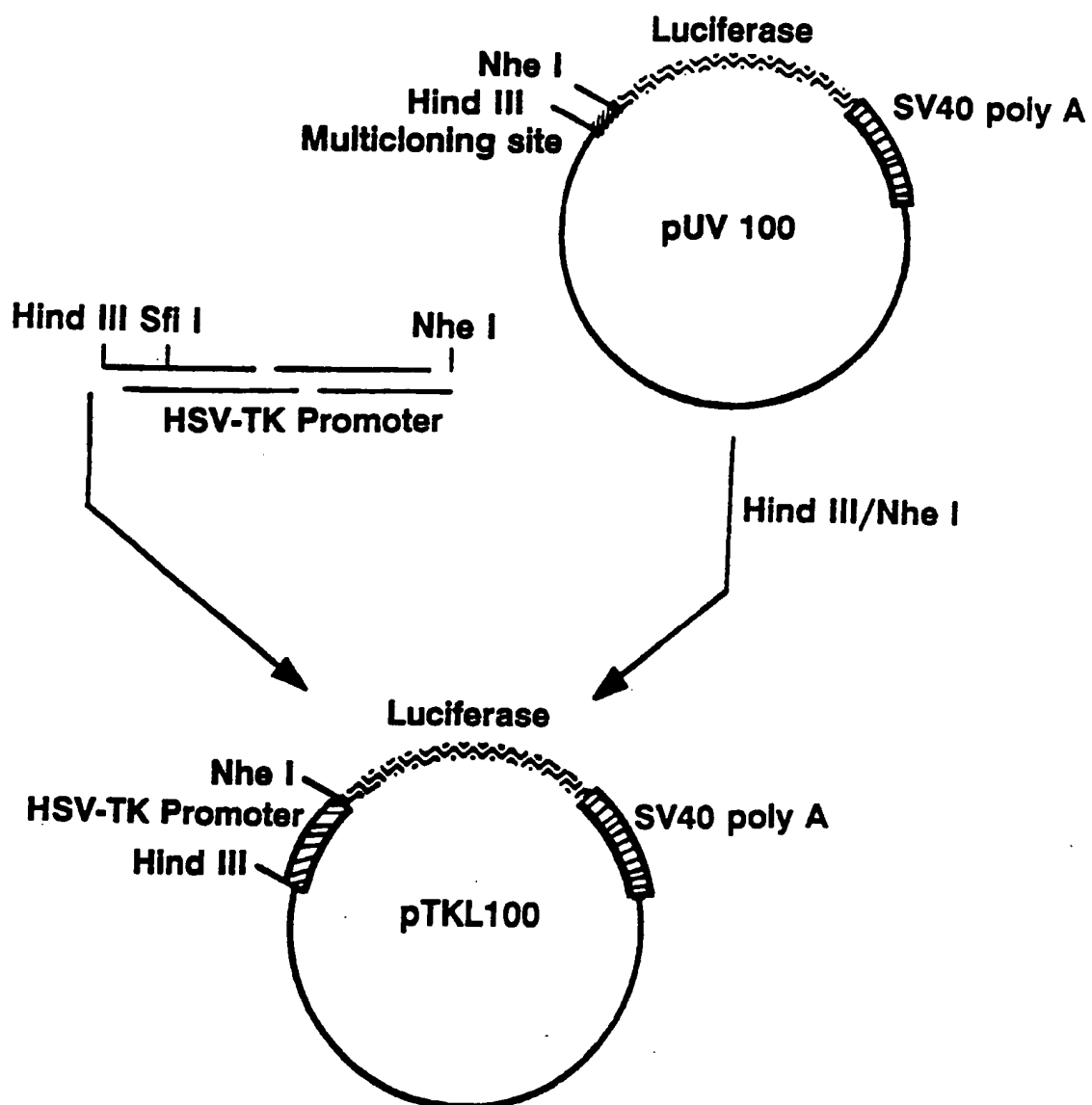
Figur 12

Constructi n f pTKNEO
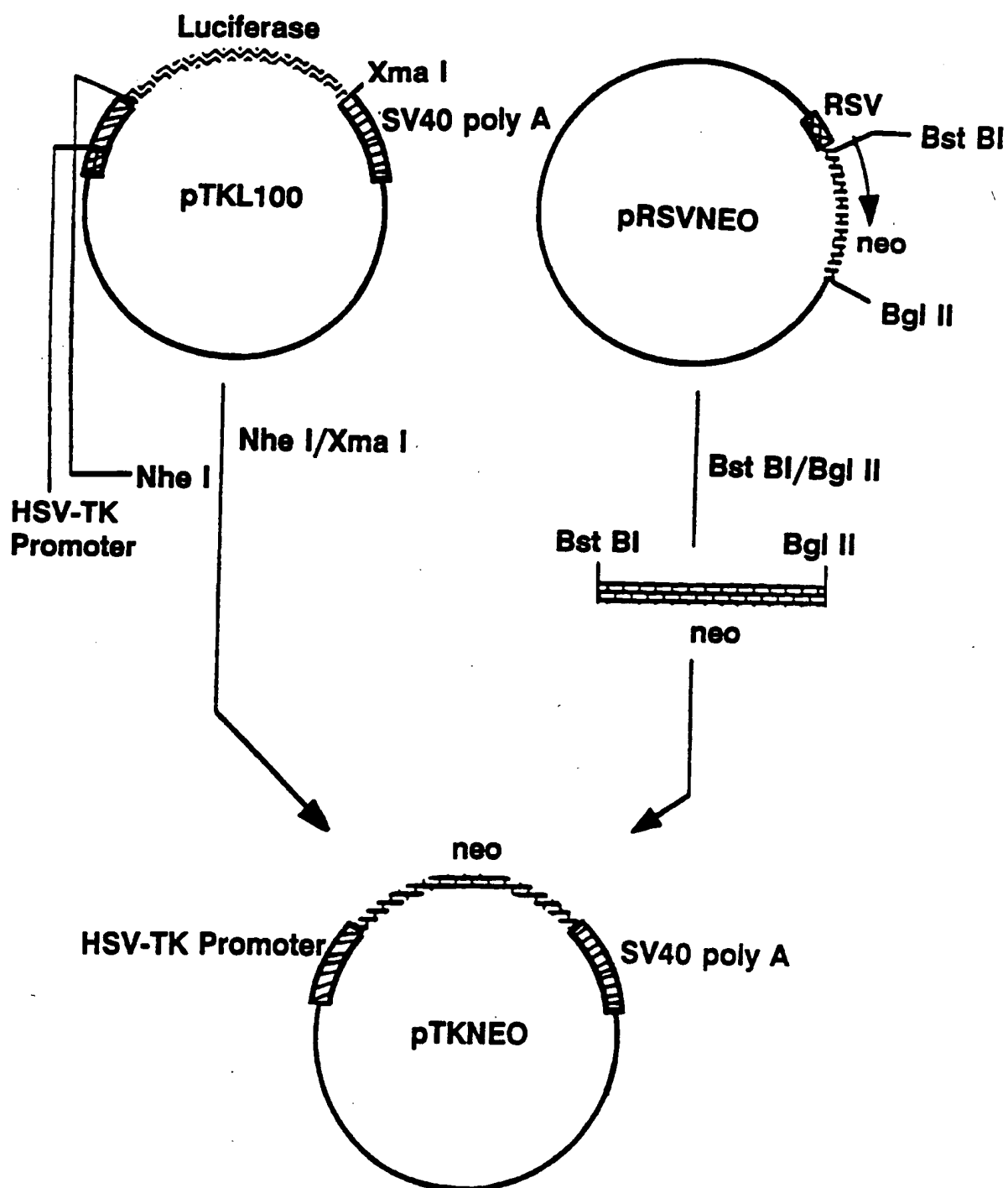
Figure 13

Construction of pTKNEO2
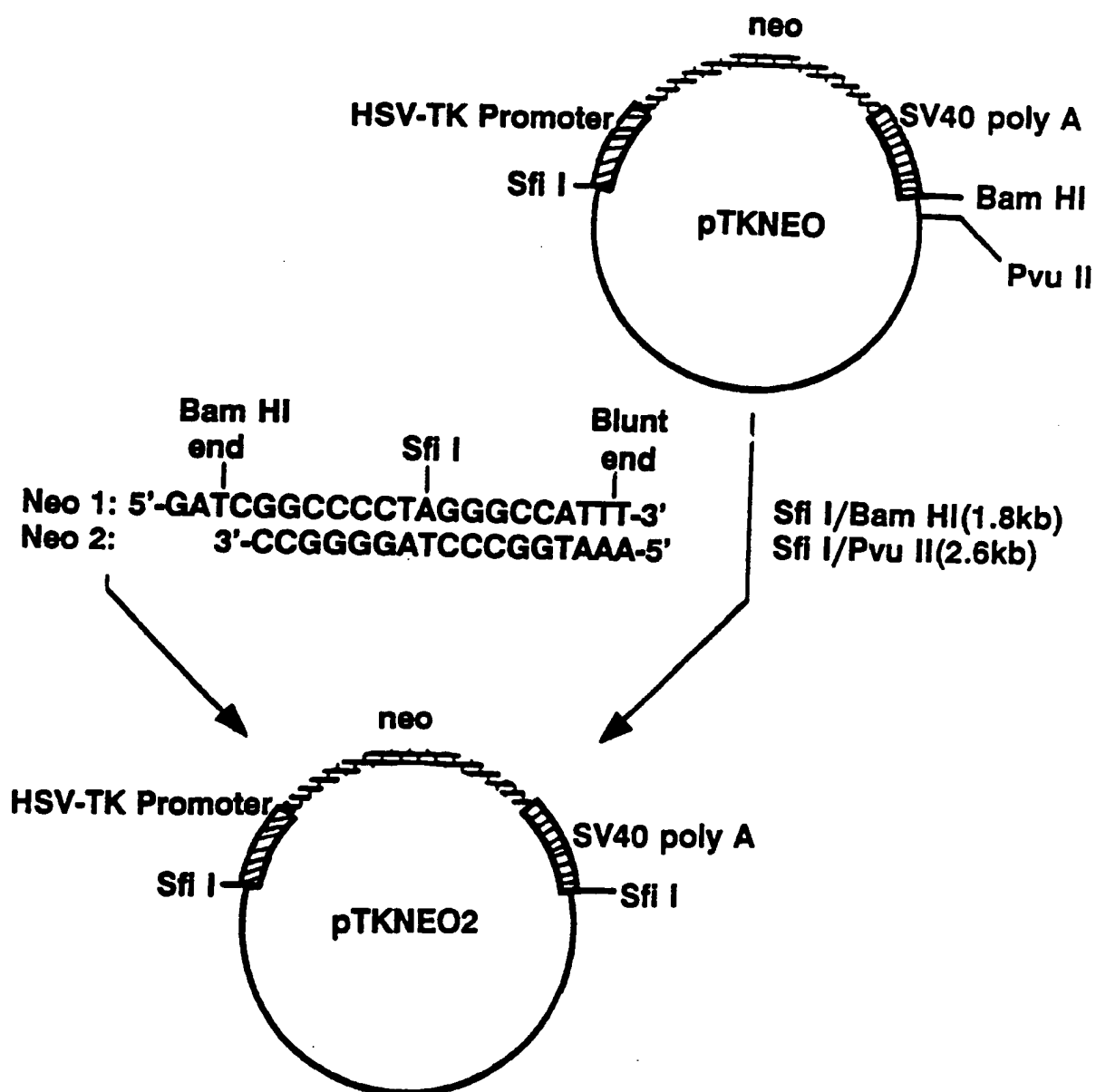
Figur 14

Construction of pTKNEO3
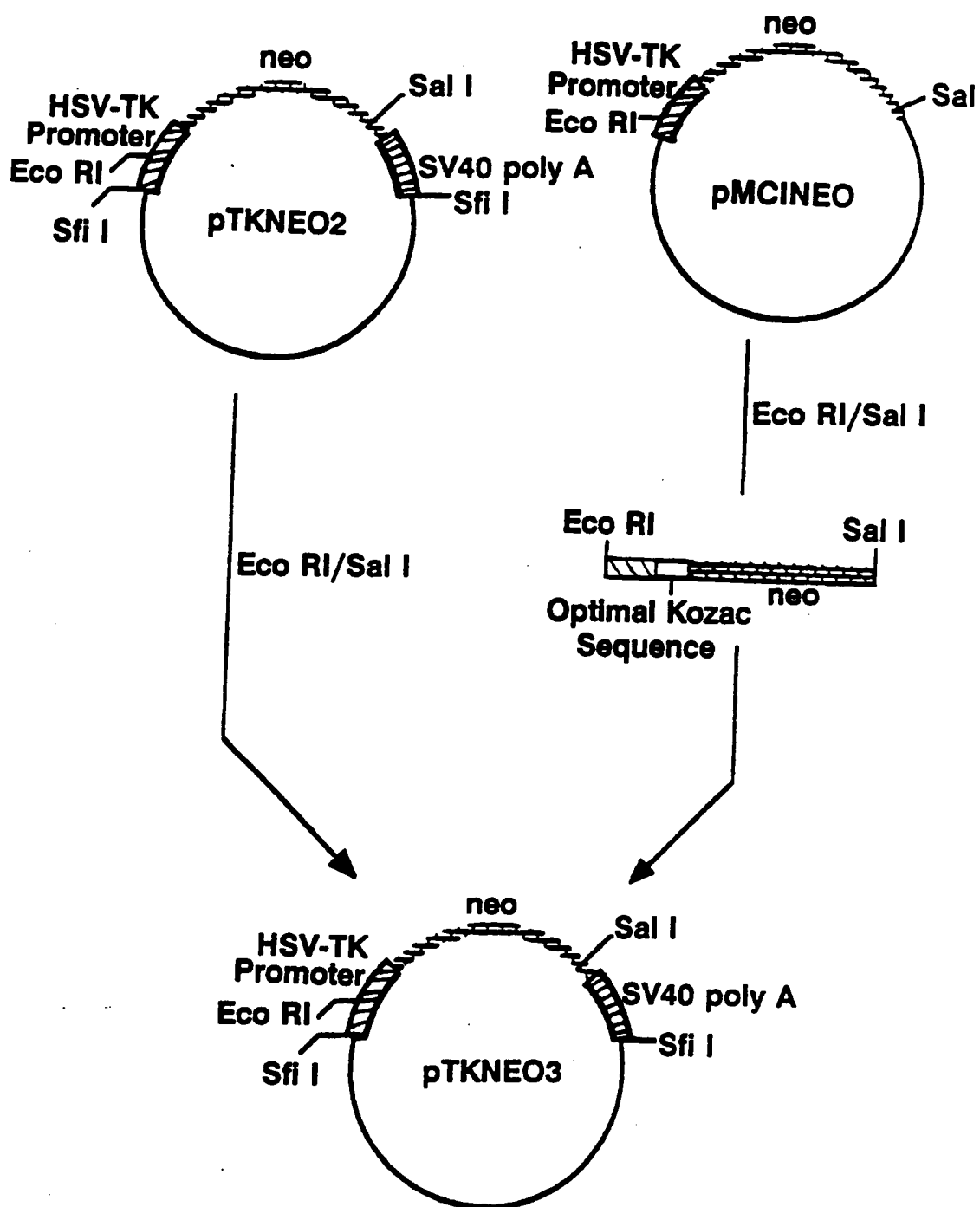
The Structure of pNEU106
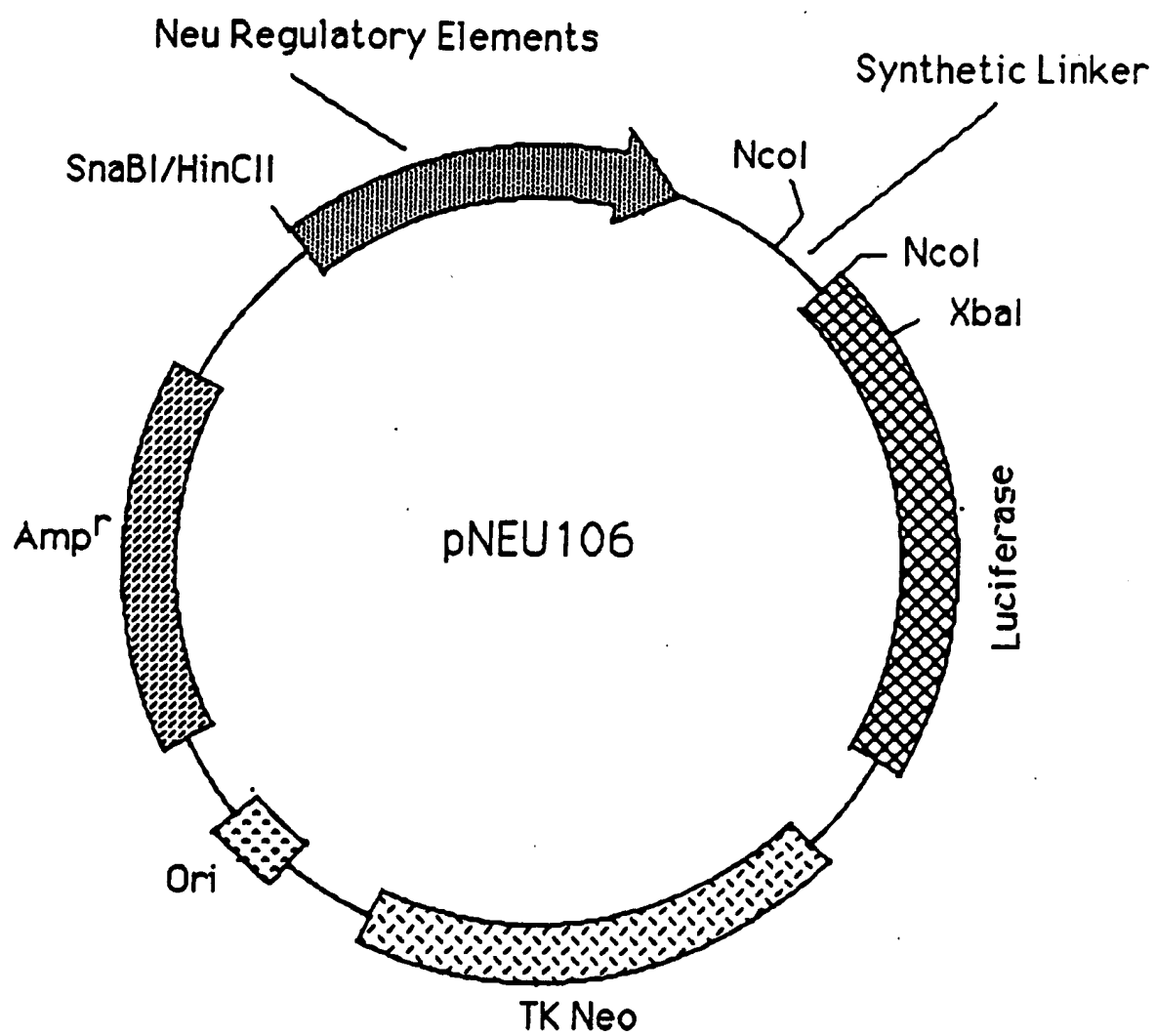
Figure 16

The Structure of pKRAS106
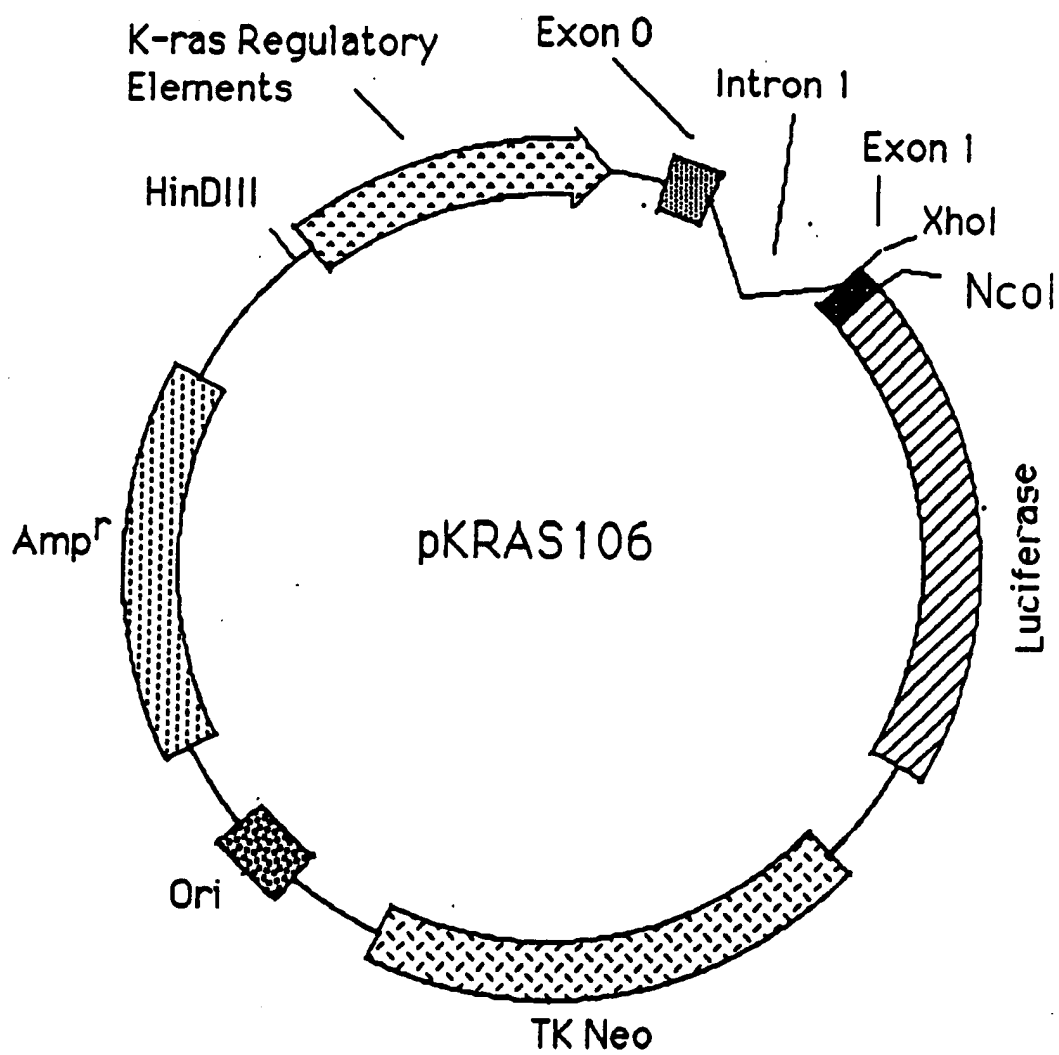
Figure 17

The Structure of pHRAS106
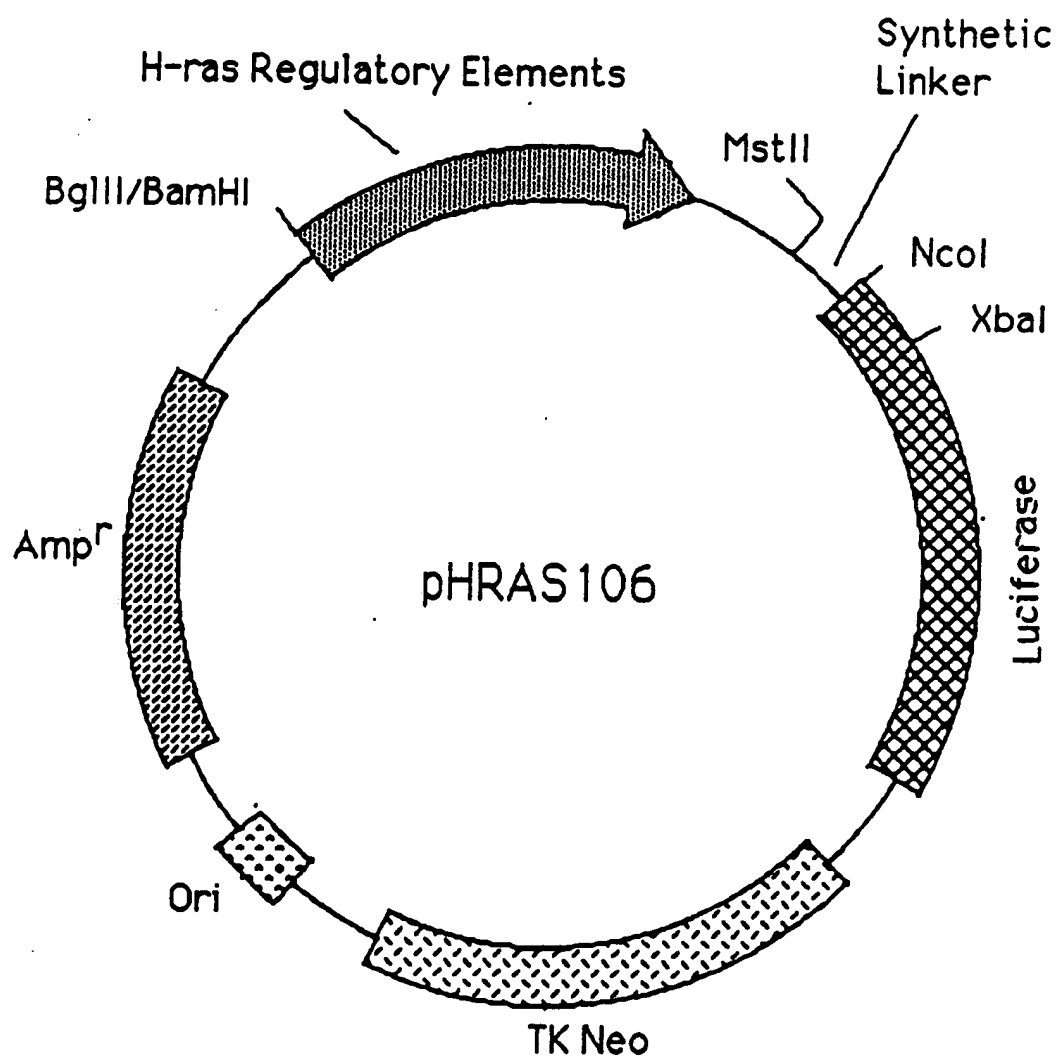
The Structure of
pNRAS106
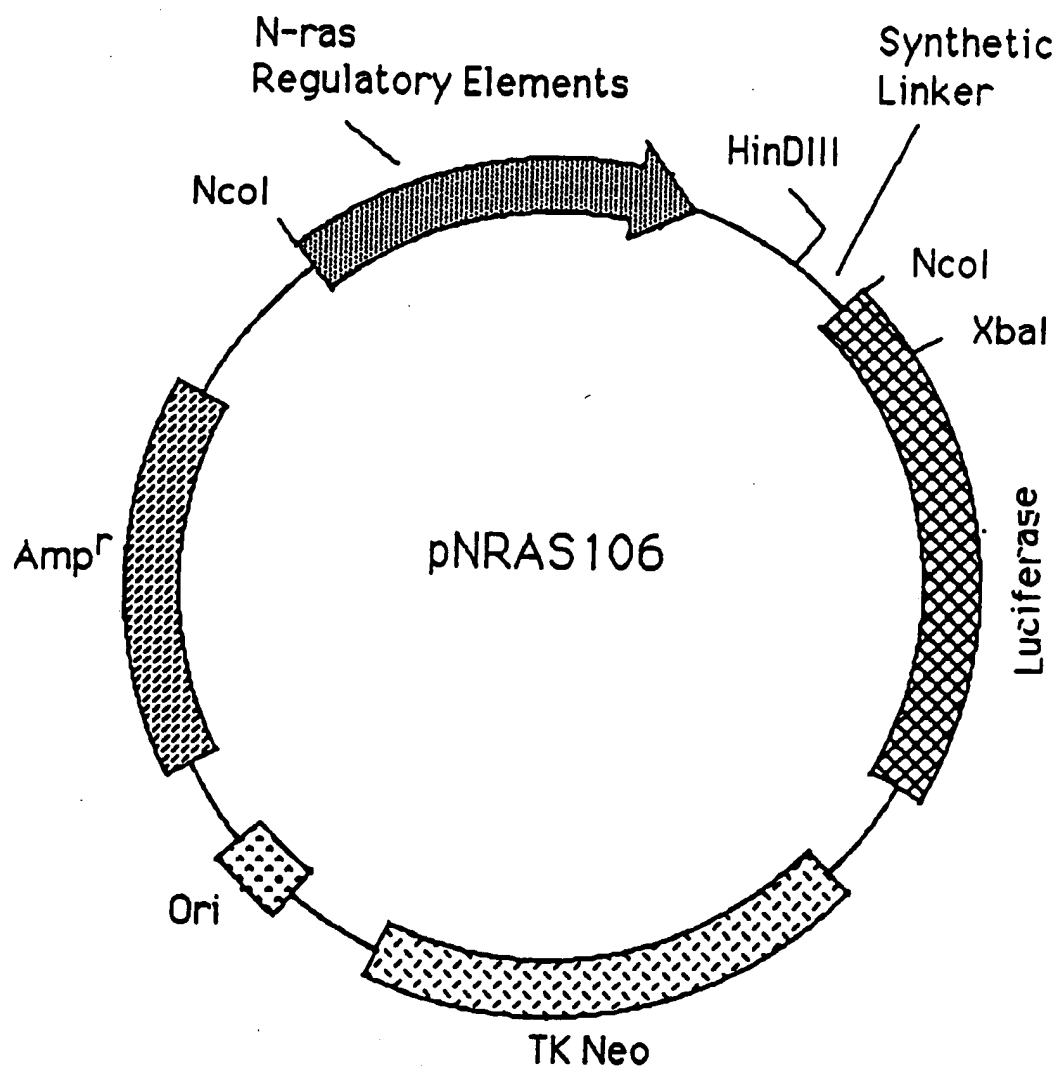
Figure 19
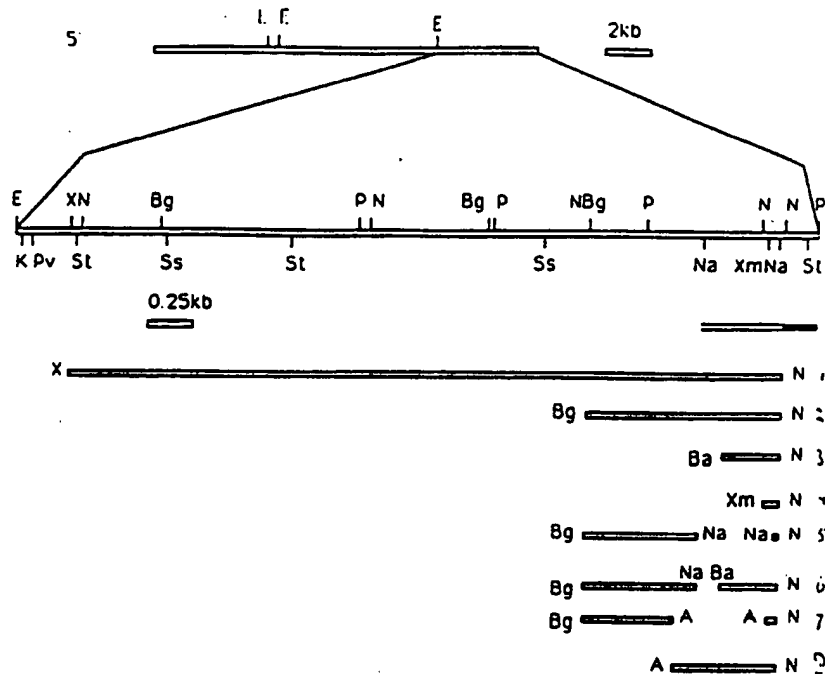
Organization of the phl promoter. Shown is an EcoRI map
of 20kb of the phl promoter. Beneath this is a
restriction enzyme map of the most 3' end containing the
most 5' 175 bp of the coding region (from NcoI to PstI)
of exon 1. The fragments numbered 1-8 have been cloned
or are being cloned into the pUV103 vector.

E=EcoRI, K=Kpnl, Pv=PvuII, X=XbaI, St=StuI, Bg=BglII,
Ss=SstI, P=PstI, N=NcoI, Na=NaeI, Ba=BalI, A=ApaI. In
the 4.4 kb EcoRI-PstI are no sites for BamHI, HindIII,
EcoRV.

Figure 20
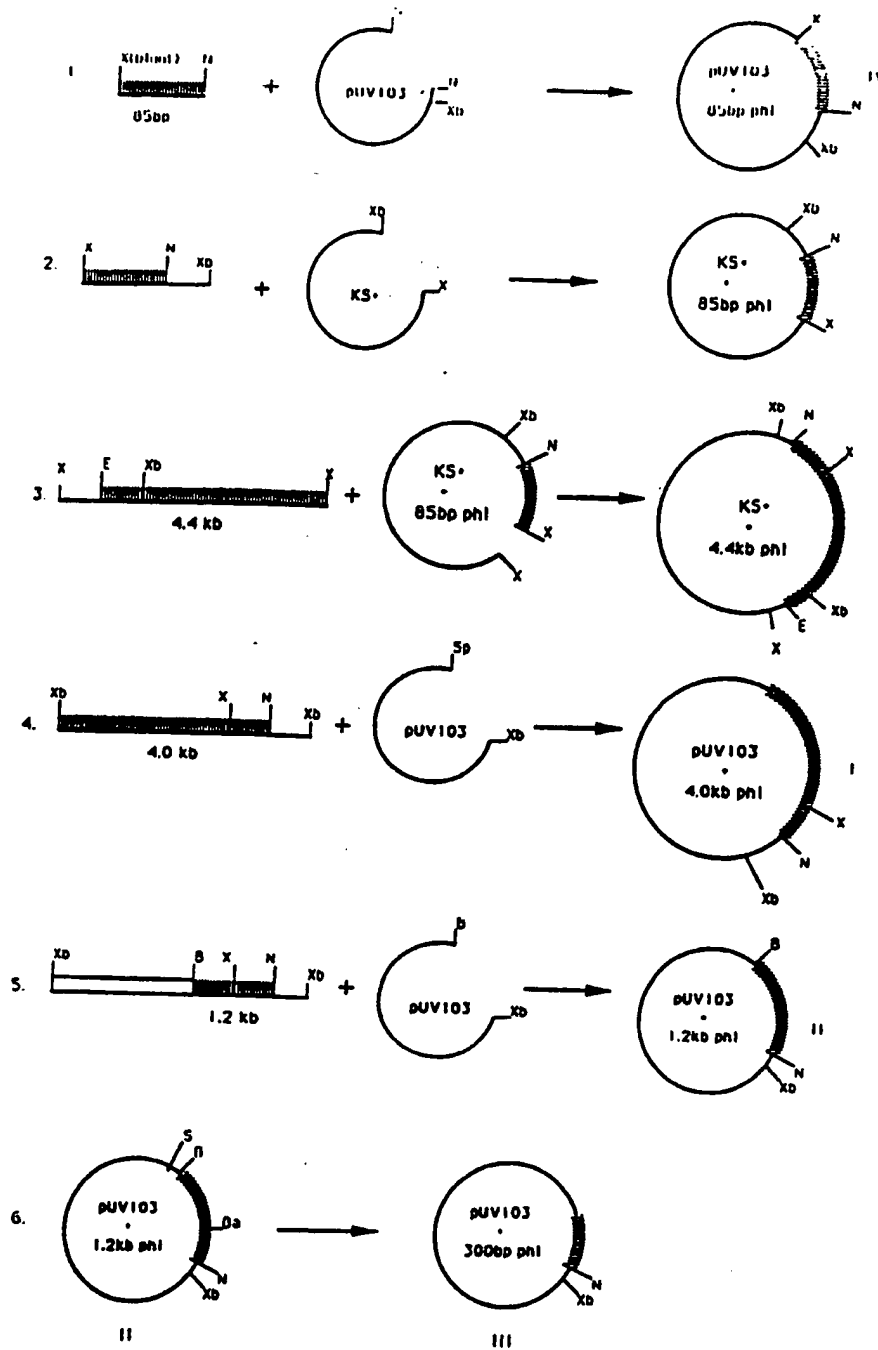
Cloning strategy of the construction of the phl promoter
luciferase constructs I through IV. X=XmaI, N=NcoI,
S=SnabI, Xb=XbaI, E=EcoRI, B=BglII and Ba=BalI.

FIGURE 21

The Structure of pP531106

p53 P1 Promoter

Beta-globin
Leader

SnaBI/EcoRI

Xbal/SnaBI

Xbal

Luciferase

Amp^r

pP531106

Ori

TK Neo
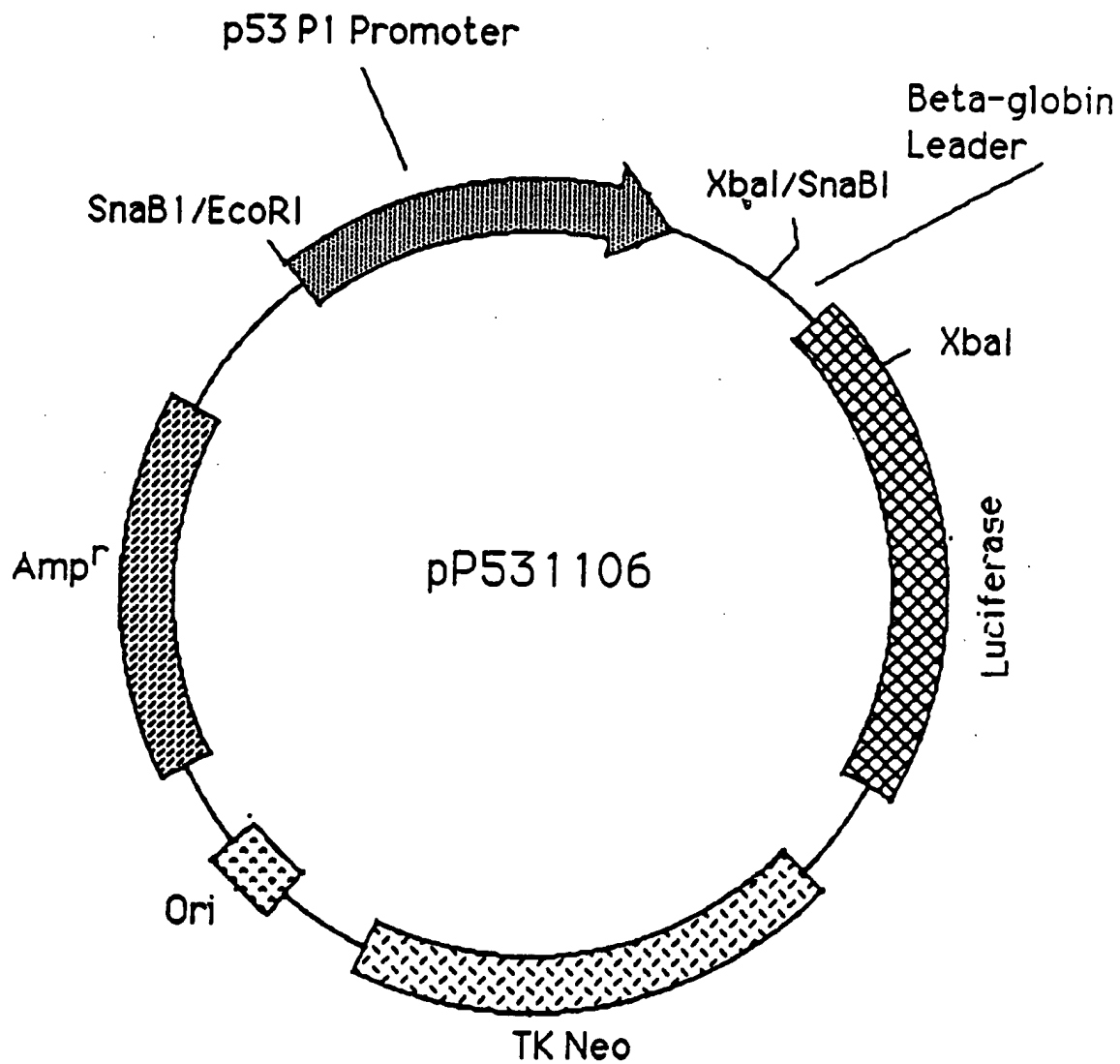
Figure 22

The Structure of pP532106
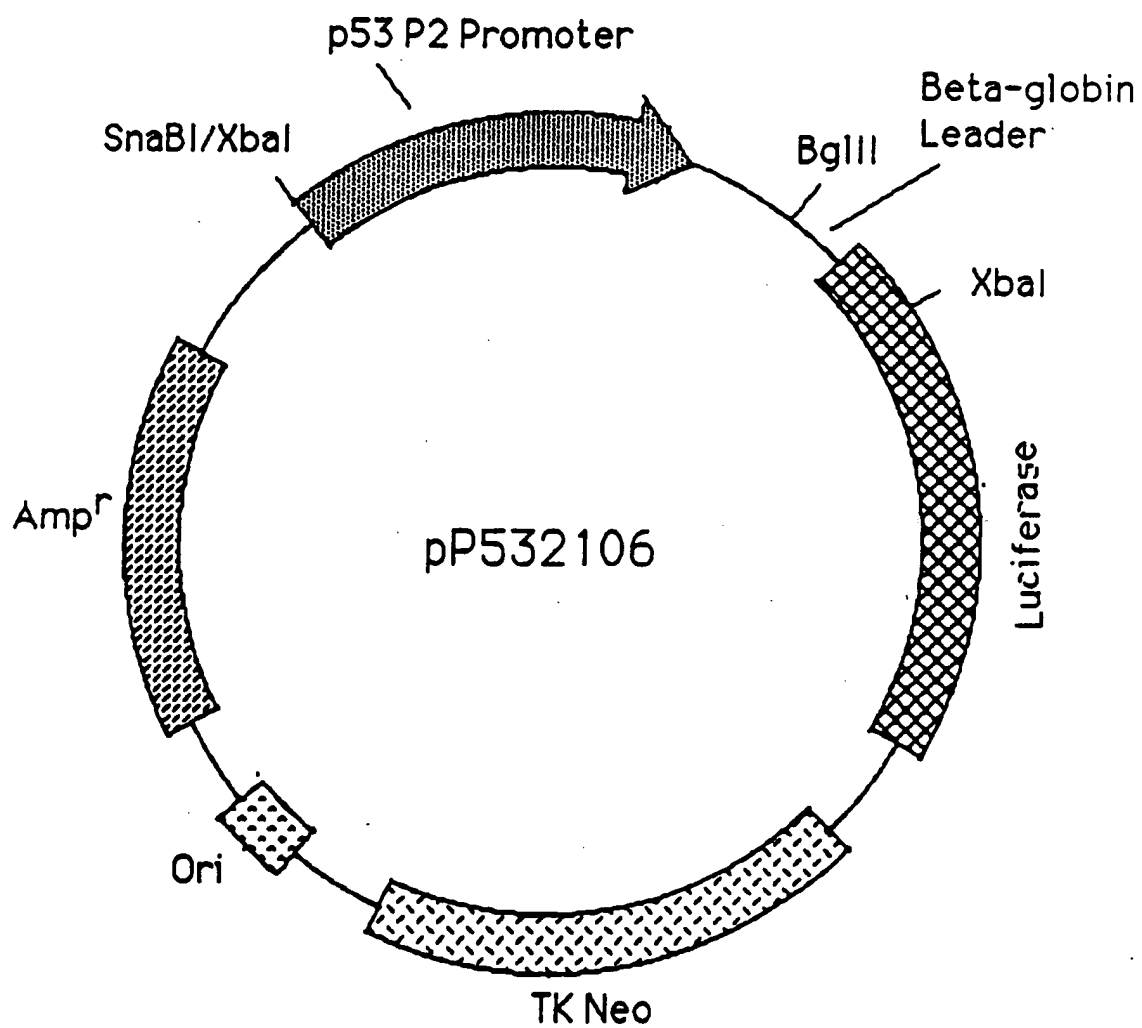
Figure 23

The Structure of pCM106
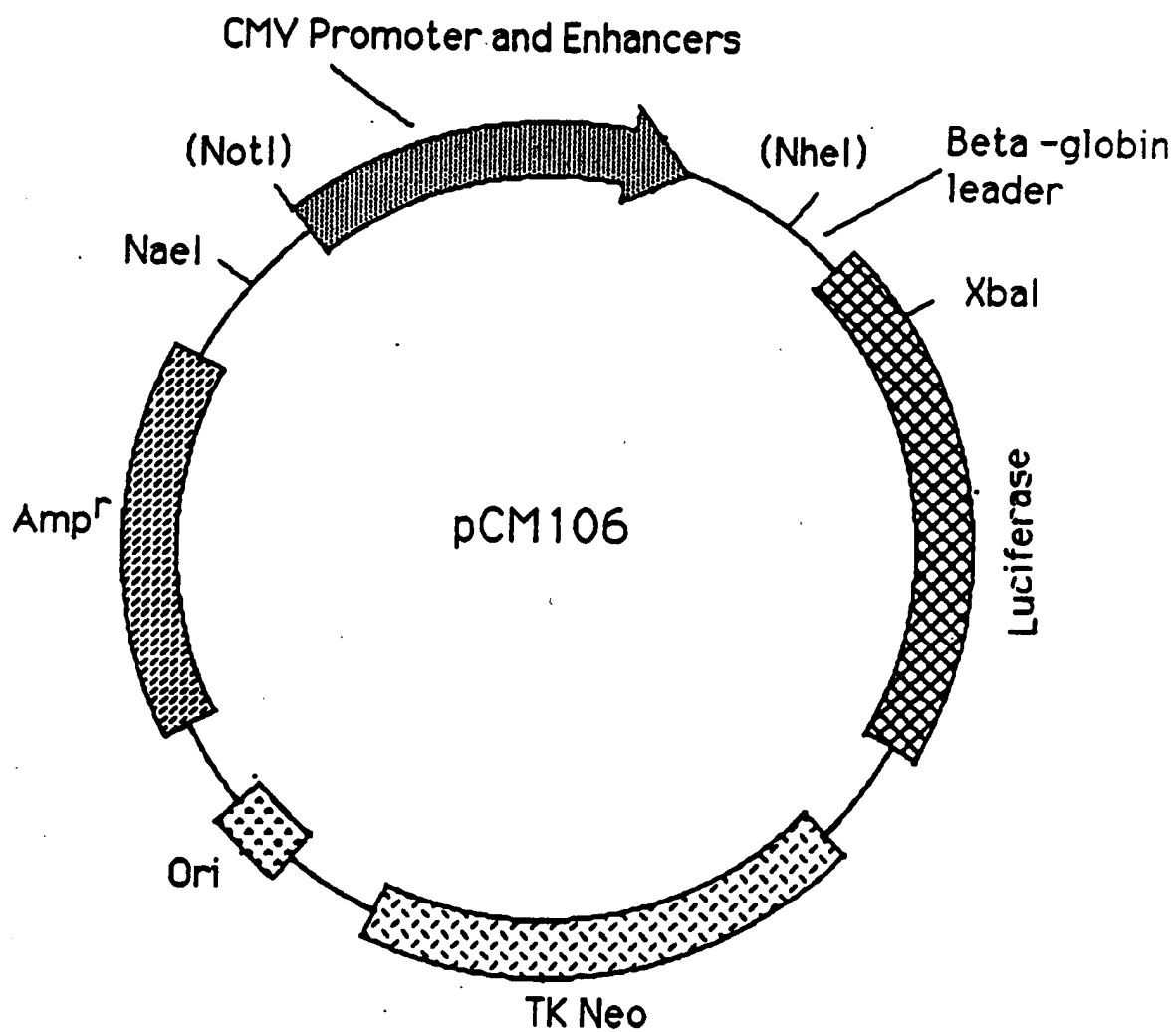
Figure 24

The Structure of
the neu Minigene
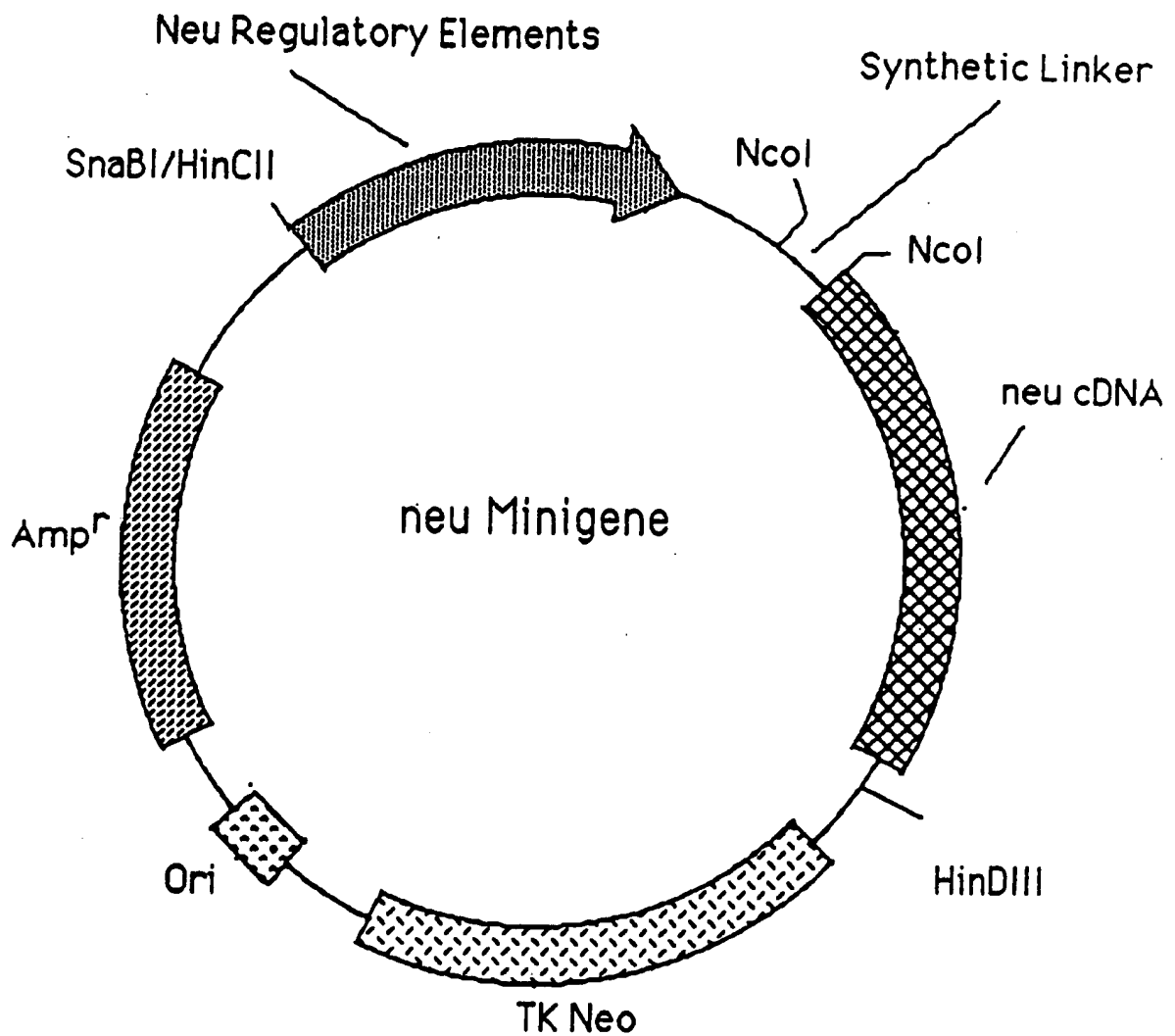
Figure 25

The Structure of the K-ras Minigene

Figure 26



Relative Intensity

Southern Blot
of Stable Clones
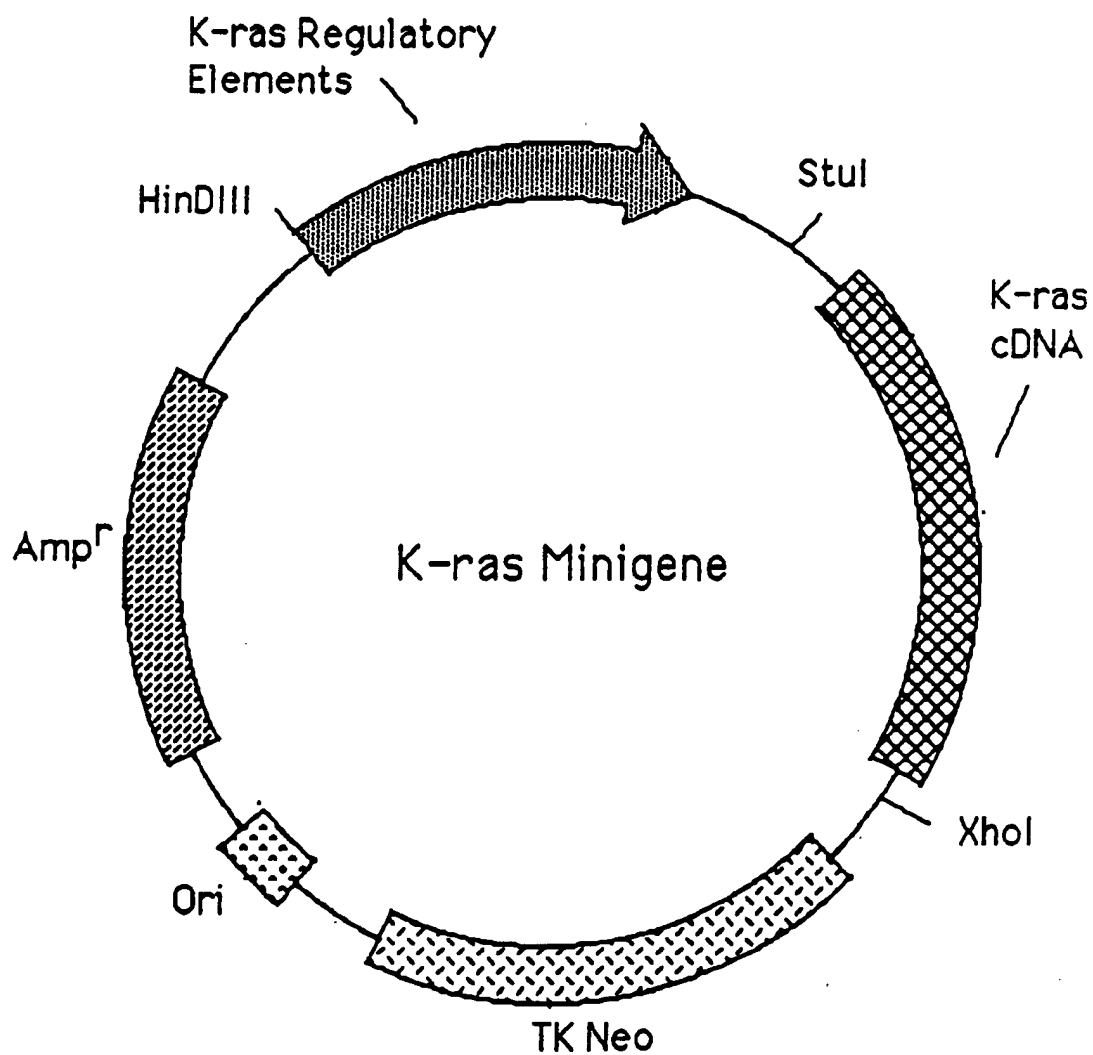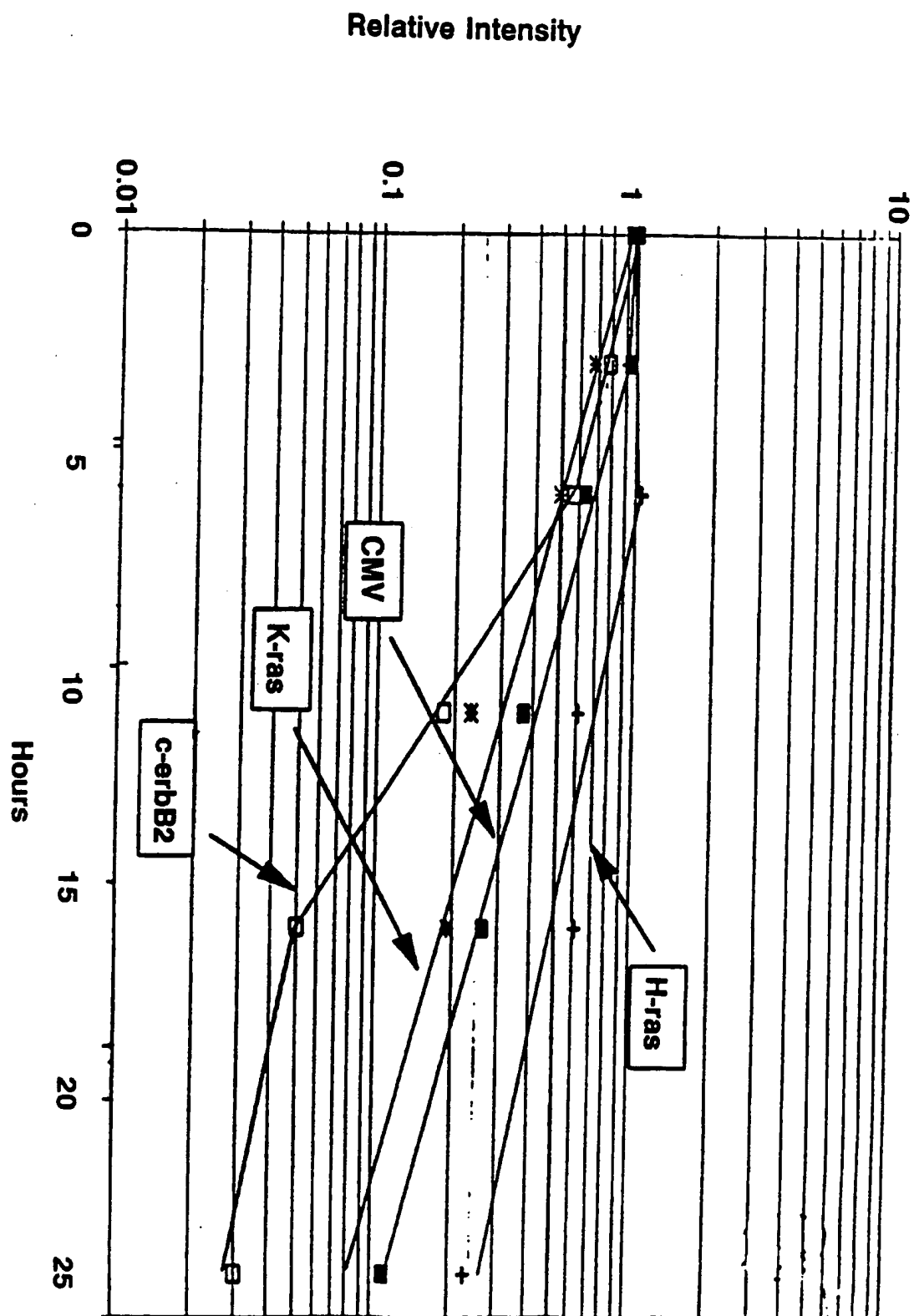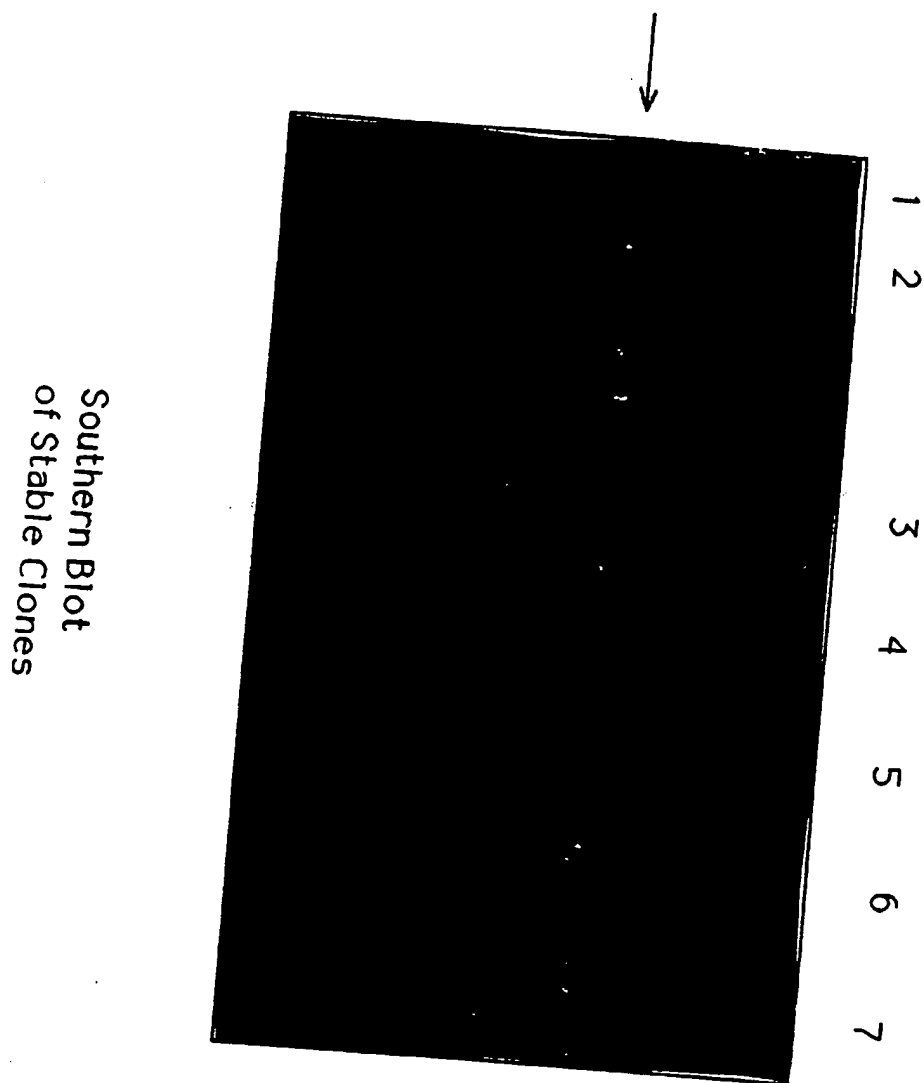
Figure 27

Figure 28
Quantitative PCR
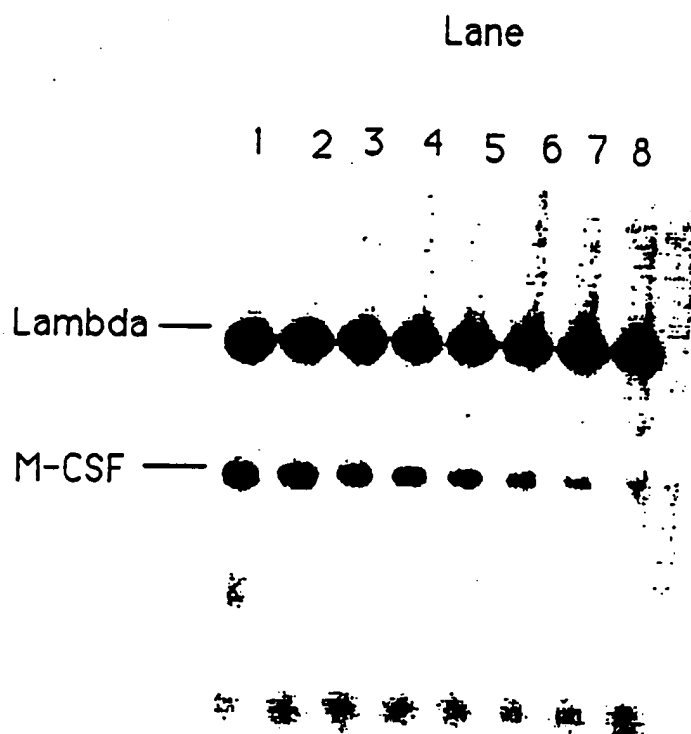
Figure 29

Quantitative PCR



Total RNA (ng)
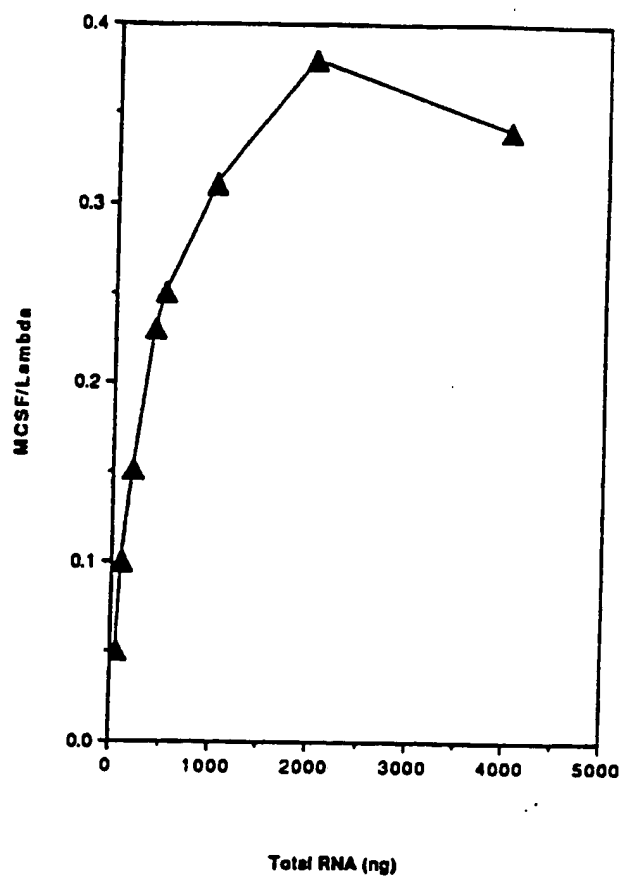
Figure 30
PCR of Oncogene mRNA
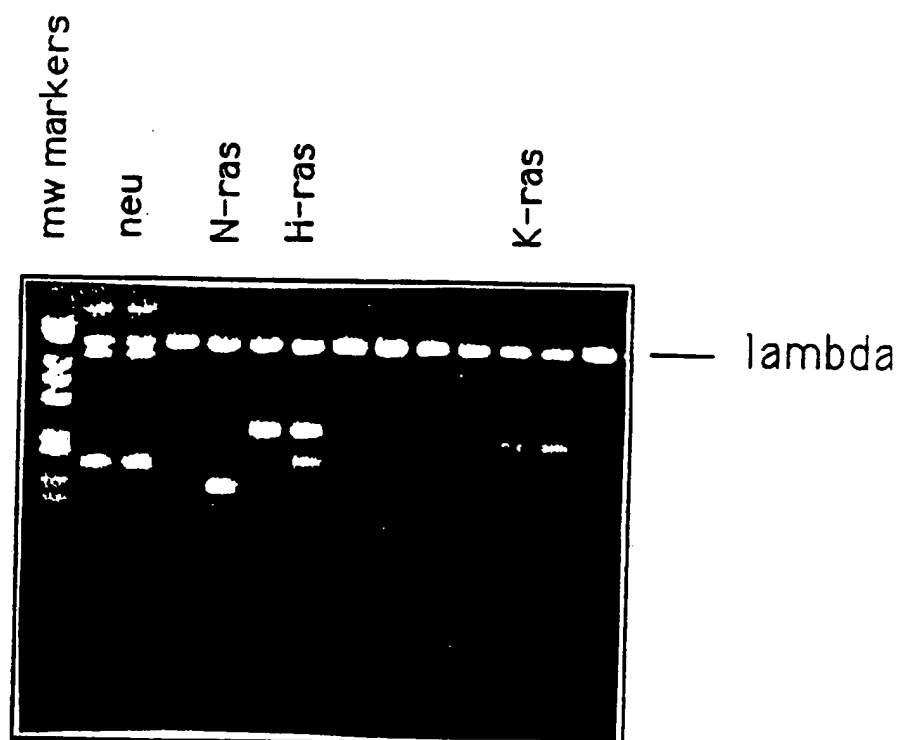
Figure 31

Ratios of Negative Controls

PF000029
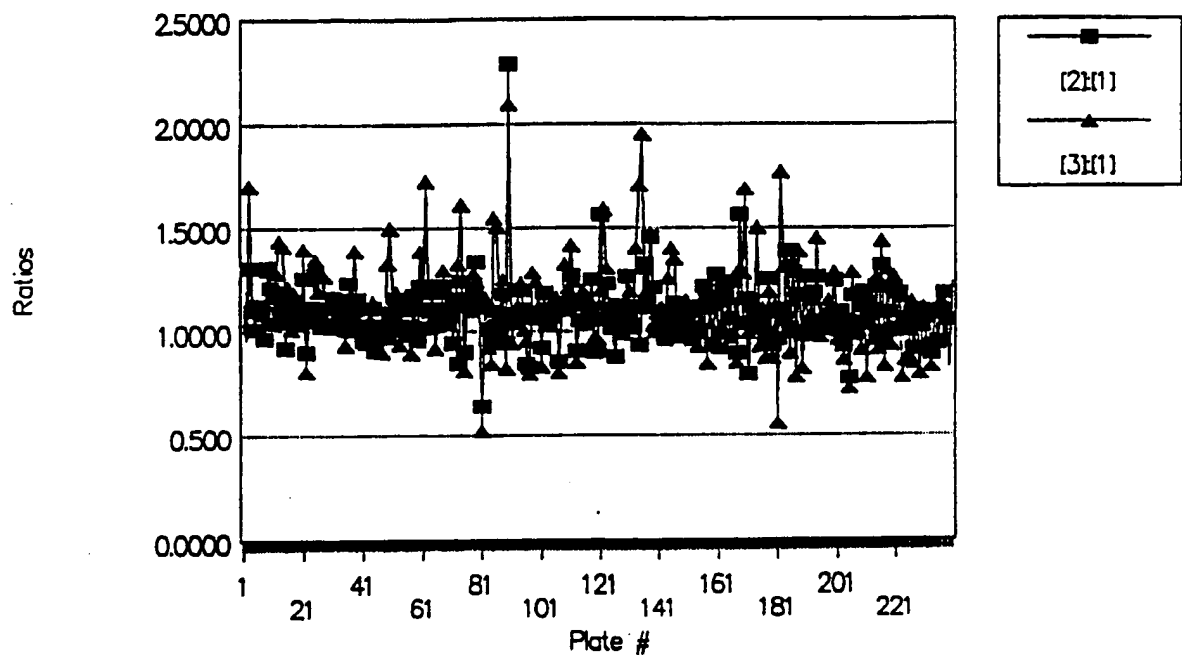
Figure 32

Average Negative RCOV

PF000029 — Avg.RCOV
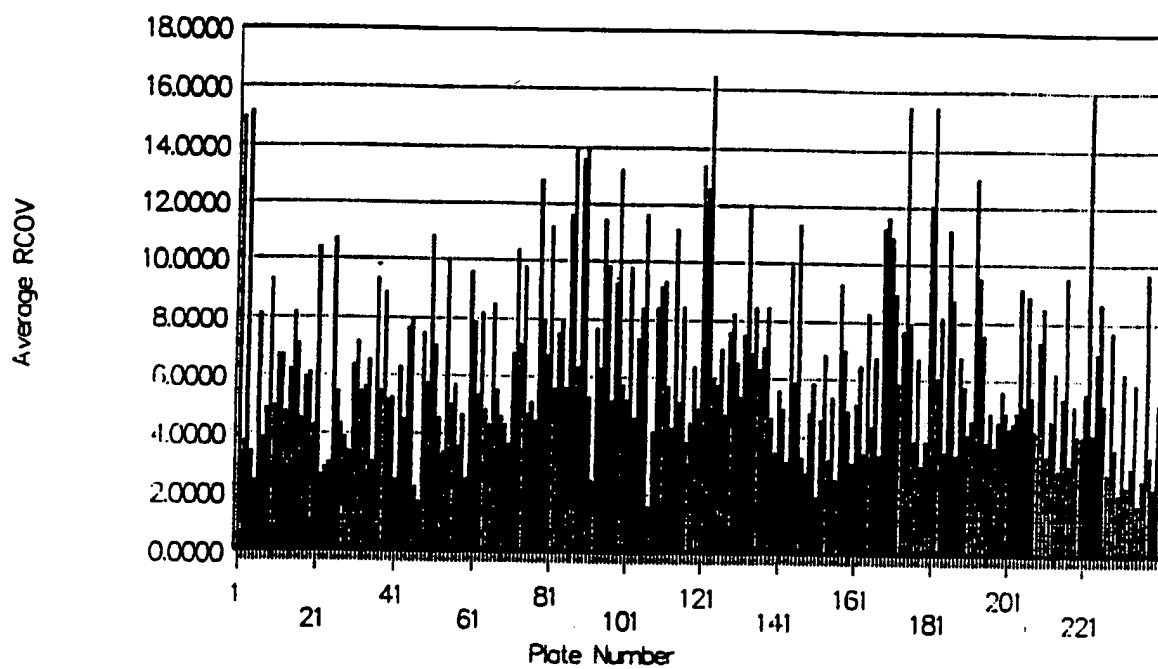
Figure 33

Average Positive Ctrl TIR RCOV

PF000029 + Avg.RCOV
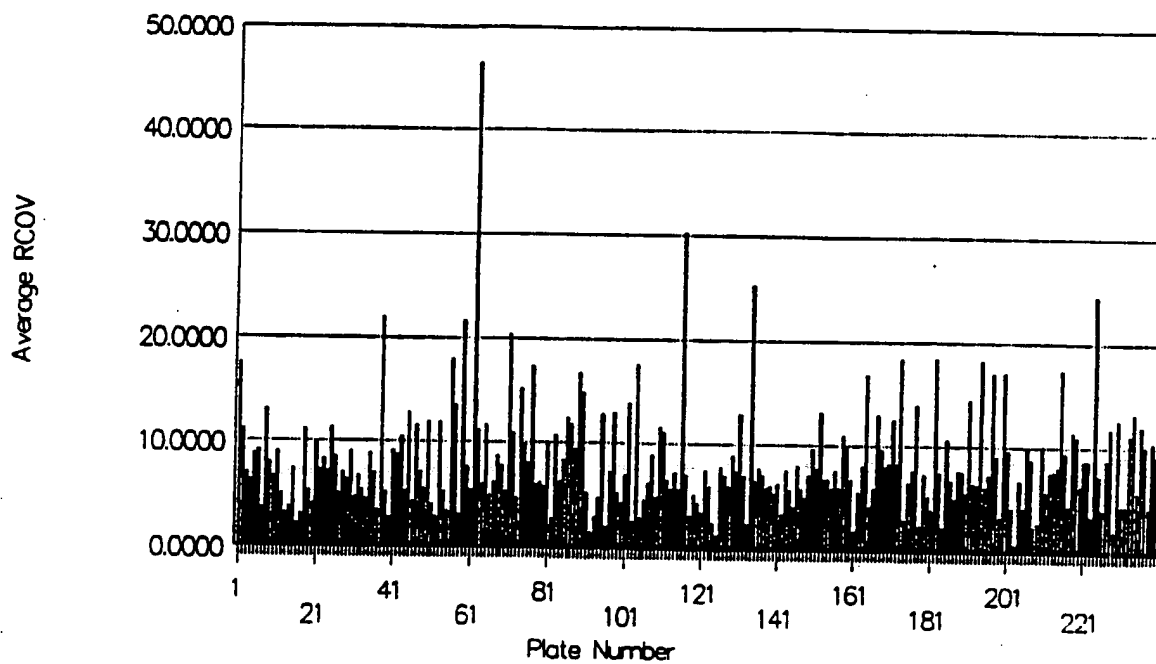
Figure 34

Positive Control TIR Signals

PF000029 Cell:ras + Medians
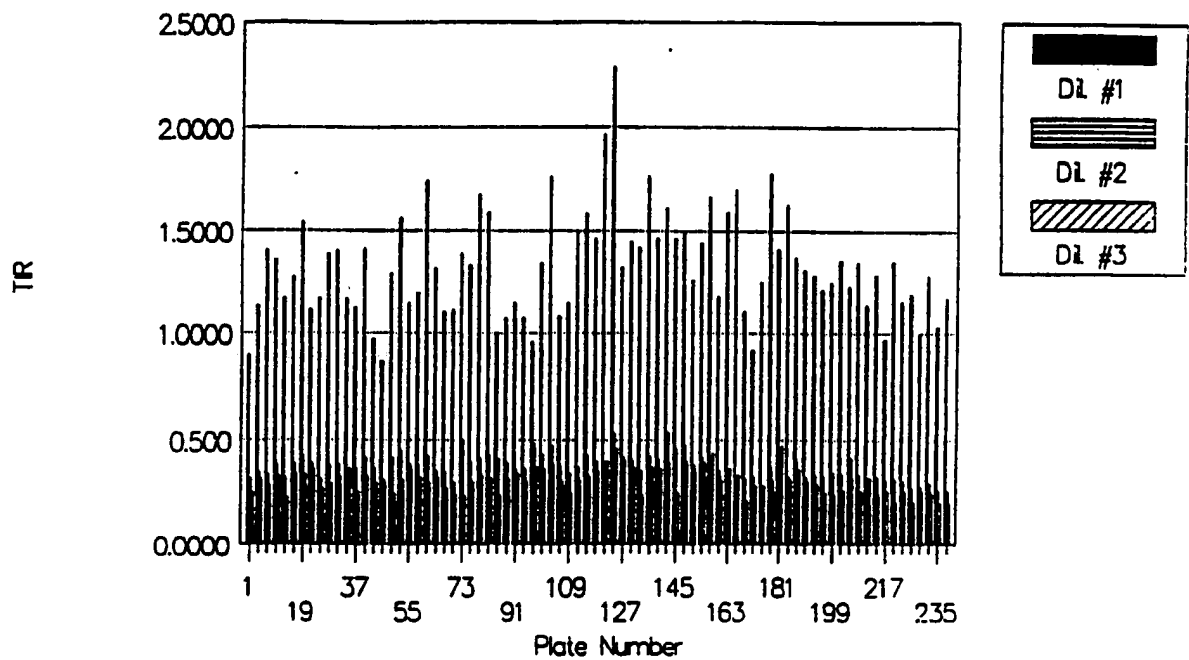
INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[3]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC (5): Please See Attached Sheet.   US CL : 435/6, 7.1, 69.1, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

II. FIELDS SEARCHED

Minimum Documentation Searched[4]

Classification System	Classification Symbols
U.S.	435/6, 7.1, 69.1, 70.1, 172.1, 172.2, 172.3, 236, 240.2, 244, 320.1

Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched[5]

Please See Attached Sheet.

III. DOCUMENTS CONSIDERED TO BE RELEVANT[14]		
Category[*]	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No.[18]
Y	US, A, 4,738,922 (Haseltine et al.) 19 April 1988. See col 1-10 and the examples.	1-23, 58-79
Y	US, A, 4,740,461 (Kaufman) 26 April 1988. See col 1-16 and the examples.	1-23, 58-79
Y	EP, A, 0,117,058 (Levinson et al., 29 August 1984. See at least the abstract and pages 5-18.	1-23, 58-79
Y	Mol. Cell. Biol. volume 7, no. 6, issued June 1987, Angel et al., "12-O-Tetradecanoyl-Phorbol-13-Acetate Induction Of The Human Collagenase Gene Is Mediated By An Inducible Enhancer Element Located In The 5'-Flanking Region", pages 2256-2266. See the abstract and the figures.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA. Volume 83, issued May 1986 Kaushansky et al., "Genomic Cloning, Characterization, Multilineage Growth Promoting Activity Of Human Granulocyte-Macrophage Colony-Stimulating Factor", pages 3101-3105. See figure 2, and page 3105.	1-23, 58-79

* Special categories of cited documents:[16]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step.
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

IV. CERTIFICATION	
Date of the Actual Completion of the International Search[2] 05 MAY 1992	Date of Mailing of this International Search Report[2] 12 MAY 1992
International Searching Authority[1] ISA/US	Signature of Authorized Officer[20] Deborah Freese for. Christopher Low

Form PCT/ISA/210 (second sheet)(May 1986) B

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

I. CLASSIFICATION OF SUBJECT MATTER:
 IPC (5):

C12P 21/00, 21/02; C12N 5/00, 7/04, 15/00, 1/38; C12Q 1/66, 1/68, 1/00

II. FIELDS SEARCHED
Other Documents Searched:

USPTO APS - USPAT, JPOABS
DIALOG - BIOSIS, CHINESE PATENT ABSTRACTS, CLAIMS/USPATENTS, INPADOC/FAMILY AND LEGAL
STATUS, WORLD PATENT ABSTRACTS.

Search Terms mammal, toxicity, toxic, chemical?, carcinogen?, oncogen?,
              transcript? breast, ovar?, panrea?, colon, lung, melanom?
              leukem?, tumor, suppress?

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
    This ISA found multiple inventions as follows:

    I.   Claims 1-32, 67-85, drawn to a method for effecting
              transcription of DNA coding for an oncogene or tumor
              suppressor, are classified in Class 435, subclasses 69.1
              and 70.1.

    II.   Claims 33-34, 36-60, 62-66, drawn to a method of testing a
              compound for effecting transcription of DNA coding for an
              oncogene or tumor suppressor by measuring the amount of a
              polypeptide, are classified in Class 435, subclass 7.1.

    III.   Claims 35-50, 61-66, drawn to a method of testing a
              compound for effecting transcription of DNA coding for an
              oncogene or tumor suppressor by measuring the amount of
              mRNA produced, are classified in Class 435, subclass 6.

	FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET	
Y	EMBO J. Volume 6, No. 9, issued September 1987, Ladner et al., "Human CSF-1: Gene Structure And Alternative Splicing Of mRNA precursors", pages 2693-2698. See at least pages 2693, 2696-2997.	1-23, 58-79
Y	EMBO J. Volume 6, No. 4, issued April 1987, Lefevre et al., "Tissue-Specific Expression Of The Human Growth Hormone Gene Is Conferred In Part By The Binding Of A Specific Trans-Acting Factor", pages 971-981. See at least page 971.	1-23, 58-79

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE[1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers _, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers _, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING[2]

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.   (Telephone Practice)

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet(2))(Rev. 4-90) ß

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Proc. Natl. Acad. Sci. USA. Volume 82, issued November 1985, Lin et al., "Cloning And Expression Of The Human Erythropoietin Gene", pages 7580-7584. See entire document.	1-23, 58-79
Y	EMBO J. Volume 5. No. 3, issued March 1986, Nagata et al., "The Chromosomal Gene Structure And Two mRNAs For Human Granulocyte Colony-Stimulating Factor", pages 575-581. See at least the abstract.	1-23, 58-79
Y	Cell Volume 47, issued 10 October 1986, Yang et al., "Human IL-3 (Multi-CSF): Identification By Expression Cloning Of A Novel Hematopoietic Growth Factor Related To Murine IL-3", pages 3-10. See figure 4, and page 6.	1-23, 58-79
Y	de Serres et al., "Chemical Mutagens. Principles And Methods For Their Detection", published 1980 by Plenum Press (New York), pages 331, and 365-473. See at least pages 331, 367-369, 377.	1-23, 58-79
Y	Science Volume 227, issued 15 March 1985, Engebrecht et al., "Measuring Gene Expression With Light", pages 1345-1347. See entire document.	1-23, 58-79
Y	Science Volume 236, issued 05 June 1987, Maniatis et al., "Regulation Of Inducible Tissue-Specific Gene Expression", pages 1237-1245. See pages 1237, 1239, 1240, 1243.	1-23, 58-79
Y	Molec. Cell. Biol. Volume 7, No. 2, issued February 1987, de Wet et al., "Firefly Luciferase Gene: Structure And Expression In Mammalian Cells", pages 725-737. See the abstract, figures 1 and 3, pages 729-734.	1-23, 58-79
Y	Exp. Hematol., vol. 16, issued 1988, Bickel et al., "Granulocyte-Macrophage Colony-Stimulating Factor Regulation In Murine T Cells And Its Relation To Cyclosporin A", pages 691 695. See entire document.	1-23, 58-79
Y	US, A, 4,601,978 (Karin) 22 July 1986, see entire document.	1-23, 58-79
Y	WO, A, 89/02472 (Shannon et al.: 23 March 1989. See entire document.	1-23, 58-79
Y	Cell, vol. 49, issued 19 June 1987, Angel et al., "Phorbol Ester-Inducible Genes Contain A Common Cis Element Recognized By A TPA-Modulated Trans-Acting Factor", pages 729-739. See entire document.	1-23, 58-79
Y	Science, vol. 230, issued 18 October 1985, Kawasaki et al., "Molecular Cloning Of A Complementary DNA Encoding Human Macrophage-Specific Colony-Stimulating Factor (CSF-1)", pages 291-296, see entire document.	1-23, 58-79
Y	Proc. Natl. Acad. Sci. USA., vol. 81, issued August 1984, Kronke et al., "Cyclosporin A Inhibits T-Cell Growth Factor Gene Expression At The Level Of mRNA Transcription", pages 5214-5218. See entire document.	1-23, 58-79

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Y	Gene Amplification (Schimke, R. T., ed.), issued 1982, Mayo et al., "Altered Regulation Of The Mouse Metallothionein I Gen Following Gene Amplification Or Transfection", pages 67-73. See entire document.	1-23, 58-79
Y,P	US, A, 5,070,012 (Nolan et al.. 13 December 1991. See entire document.	24-51, 52-57
Y	US, A, 4,981,783 (Augenlicht 01 January 1991 see entire document.	24-51, 52-57
Y	US, A, 4,806,463 (Goodchild et al.) 21 February 1989. See entire document.	24-41, 52-57
Y	US, A, 4,861,709 (Ulitzur et al.), 29 August 1989. See entire document.	24-41, 52-57
Y	US, A, 4,935,363 (Brown et al.), 19 June 1990. See entire document.	33-66
Y	Nature, vol. 346, issued 16 August 1990, Standaert et al., "Molecular Cloning And Overexpression Of The Human FK506-Binding Protein FKBP", pages 671-674. See entire document.	33-66
Y	J. Immunol., vol. 143, no. 2, issued 15 July 1989, Tocci et al., "The Immunosupprressant FK506 Selectively Inhibits Expression Of Early T Cell Activation Genes", pages 718-726. See entire document.	33-66
Y	Bio/Technol., vol. 7, issued March 1989, Ratner, "Can The Antisense Message Be Delivered?", page 207. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 6, issued June 1989, Cao et al., "A Simple And Inexpensive System To Amplify DNA By PCR", pages 566-567. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 6 issued June 1989, Lim et al., "A Simple Assay For DNA Transfection By Incubation Of The Cells In Culture Dishes With Substrates For Beta-Galactosidase", pages 576-579. See entire document.	33-66
Y	Bio/Technol., vol. 7, issued March 1989, McCall et al., "Biotherapy: A New Dimension In Cancer Treatment", pages 231-240. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Brenner et al., "Message Amplification Phenotyping (MAPPing): A Technique To Simultaneously Measure Multiple mRNAs From Small Numbers Of Cells", pages 1096-1103. See entire document.	33-66
Y	US, A, 4,761,371 (Bell et al.) 02 August 1988. See entire document.	1-85
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989, Munjaal et al., "In Situ Detection Of Progesterone Receptor mRNA In The Chicken Oviduct Using Probe-On Slides", pages 1104-1108. See entire document.	33-66

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No.[18]
Y	Bio/T chniques, vol. 7, no. 10, issued November/December 1989, Brasier et al., "Optimized Use Of The Firefly Luciferase Assay As A Reporter Gene In Mammalian Cell Lines", pages 1116-1122. See entire document.	33-66
Y	Bio/Techniques, vol. 7, no. 10, issued November/December 1989. Slack et al., "Application Of The Multiscreen System To Cytokine Radioreceptor Assays", pages 1132-1138. See entire document.	33-66
Y	Bio/Techniques, vol. 8, no. 1, issued January 1990, Rao et al., "A Quantitative Assay For ß-D-Glucuronidase (GUS) Using Microtiter Plates", pages 38-40. See entire document.	33-66
Y	Bio/Techniques, vol. 8, no. 3, issued March 1990, Willingham et al., "A Reversible Multi-Well Chamber For Incubation Of Cultured Cells With Small Volumes: Application To Screening Of Hybridoma Fusions Using Immunofluorescence Microscopy", pages 320-324. See entire document.	33-66
Y	Bio/Techniques, vol. 9, no. 4, issued October 1990, Pons et al., "A New Cellular Model Of Response To Estrogens: A Bioluminescent Test To Characterize (Anti)Estrogen Molecules", pages 450-459. See entire document.	33-66
Y	US, A, 4,740,463 (Weinberg et al.) 26 April 1988. See entire document.	1-85
Y	US, A, 4,885,238 (Reddel et al.) 05 December 1989. See entire document.	1-85
Y	US, A, 4,699,877 (Cline et al.) 13 October 1987. See entire document.	33-66
Y	US, A, 4,736,866 (Leder et al.) 12 April 1988. See entire document.	1-85
Y	US, A, 4,535,058 (Weinberg et al) 13 August 1985. See entire document.	33-66